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PREFACE

The research presented in this thesis was done during my part-time PhD employment at Aalborg University Hospital in the years 2012-2017 in parallel with my specialist training in clinical biochemistry. The performed studies fall within the scope of two current target areas of research at Aalborg University Hospital, namely atrial fibrillation (AF) and extracellular vesicles (EVs).

In 2009, a group of experienced researchers at Aalborg University Hospital, including my main supervisor, professor Søren Risom Kristensen and co-supervisor, professor Lars Hvilsted Rasmussen, formed the Atrial Fibrillation Study Group (AF Study Group) and established the project "Atrial Fibrillation – an epidemic. From genes and lifestyle to prevention and treatment". In collaboration with international colleagues, the AF Study Group has since its foundation conducted studies on several aspects of atrial fibrillation and contiguous topics.

In 2010 a burgeoning international interest in cell-derived EVs as mediators of various physiological and pathological bodily processes, including thrombosis, occasioned the arrangement of the first international meeting on "Microvesicles and Nanovesicles in Health and Disease" at Magdalen College, Oxford, UK, which I attended in company with professor Søren Risom Kristensen. One of the matters presented at the meeting, was the procoagulant potential of some EVs, among these subtypes exposing the procoagulant transmembrane protein termed tissue factor (TF).

My PhD project is rooted in the AF Study Group, and its onset was an interest in the mechanisms causing thrombus formation in the left atrial appendage (LAA) of the heart and consequent thromboembolism and stroke as a common and serious complication in patients with AF. Anatomical and functional changes, including TF production, in the endocardium of the LAA in AF patients had been described in the literature. It was generally accepted that EVs were released from the plasma membrane of various cell types as a response to cell stress. Therefore, we nominated the potential release of procoagulant EVs from the LAA endothelium as one of the conceivable mechanisms of hypercoagulability in the LAA in AF patients. This hypothesis would become the objective for much of the work presented in this thesis, and challenges related to measurement of the heterogenous group of analytes collectively named EVs led us on a byway, which, for long stretches, would constitute the main route of the project.

At the first annual meeting of the International Society of Extracellular Vesicles (ISEV), held in Gothenburg in April 2012, alongside a catching positivity towards the potential of EVs as future diagnostic and prognostic biomarkers, there was a common recognition of the necessity of considerable development in techniques for

EV isolation and analysis in order to fulfil that potential. As of 2017, the interest in EVs among researchers in various medical fields is still growing, and many associations between medical conditions and detected EVs described in the literature hold out interesting prospects of clinical diagnostic applications of EV analysis. However, technical limitations regarding the preparation and analysis of EVs in compositionally complex body fluids still hamper actual implementation of EVs as valuable analytes in a clinical setting.

In our research, we have taken particular interest in EVs present in blood plasma. This has entailed considerations, and accordingly studies, on different factors causing variation in the results, not least the analytical interference caused by another type of particles present in this medium, namely lipoproteins. We hope that our investigations can contribute constructively to the body of knowledge in this field.

I want to thank all my supervisors and all co-authors of the papers that form the basis for this thesis for their participation in this project. A special thanks to my main supervisor, Søren Risom Kristensen, for many constructive discussions, including a great amount of professional back-and-forth during the paper writing. Likewise, a special thanks to professor Jan Jesper Andreasen for including patients and making sample collection for study 4 possible. In that connection, I would also like to thank the other surgeons at the Department of Cardiothoracic Surgery along with the anesthesiologists and surgical nurses participating in sample collection in the operating room. Furthermore, I am grateful for the assistance that I received from several colleagues at the Department of Clinical Biochemistry regarding sample analysis, information technology, and taking over daily tasks during busy periods of the project. Finally, I want to thank my ever-supportive friends and family, particularly my wonderful wife, Katrine, and our cherished children, Mikkel, Mynte, Rasmus, and Mads, for being a solid source of encouragement and inspiration.

Morten Mørk

READING GUIDE

The thesis is based on four studies documented in each their paper. The *list of papers* is given on the following page.

Then follows a *background* chapter, which is structured into three sections, each introducing one of the topics mentioned in the thesis title, namely “atrial fibrillation”, “thrombosis”, and “extracellular vesicles”.

With the background chapter as a foundation, the ideas, approaches, and main findings of the four studies are collectively presented and discussed along with further perspectives, in the *aims and hypotheses*, *methods*, *result*s, and *discussion* chapters.

The content of the thesis is then summarized in an *English* and a *Danish summary*.

Abbreviations used in this thesis are introduced in parantheses, and for look-up a full *list of abbreviations* is given prior to the *list of references*.

LIST OF PAPERS

Paper 1

Preanalytical, analytical, and biological variation of blood plasma submicron particle levels measured with nanoparticle tracking analysis and tunable resistive pulse sensing.
Morten Mørk, Shona Pedersen, Jaco Botha, Sigrid Marie Lund, and Søren R. Kristensen. *Scandinavian Journal of Clinical & Laboratory Investigation, Vol. 76, No. 5, 2016, p. 349-360.*
NB: A supplementary note on technical considerations was published along with paper 1.

Paper 2

Prospects and limitations of antibody-mediated clearing of lipoproteins from blood plasma prior to nanoparticle tracking analysis of extracellular vesicles.
Morten Mørk, Aase Handberg, Shona Pedersen, Malene M. Jørgensen, Rikke Bæk, Morten K. Nielsen, and Søren R. Kristensen. *Journal of Extracellular Vesicles, Vol. 6:1, 2017.*

Paper 3

Postprandial increase in blood plasma levels of tissue factor-bearing (and other) microvesicles measured by flow cytometry – fact or artefact?
Morten Mørk, Morten H. Nielsen, Malene M. Jørgensen, Rikke Bæk, Shona Pedersen, and Søren R. Kristensen. *Submitted*.

Paper 4

Elevated blood plasma levels of tissue factor-bearing extracellular vesicles in patients with atrial fibrillation.
Morten Mørk, Jan J. Andreasen, Lars H. Rasmussen, Gregory Y.H. Lip, Shona Pedersen, Rikke Bæk, Malene M. Jørgensen, and Søren R. Kristensen. *Submitted*.

BACKGROUND

ATRIAL FIBRILLATION

DISCOVERY

"When the pulse is irregular and tremulous and the beats occur at intervals, then the impulse of life fades; when the pulse is slender (smaller than feeble, but still perceptible, thin like a silk thread), then the impulse of life is small."

The quote above is from the ancient Chinese medical text, Huang Di Nei Jing Su Wen ("The Yellow Emperor's Inner Canon"), written hundreds of years before common era[1]. Notes on health issues related to a palpable irregular pulse, most likely caused by the heart rhythm disorder, we now know as atrial fibrillation (AF), were also made by other prominent historical medical practitioners, including Moses Maimonides in the 12th century and William Stokes in the 19th century[2].

In the first decade of the 20th century, new technology paved the way for scientific advances in the understanding of the nature of the characteristic sustained, irregular pulse termed "pulsus irregularis perpetuus" by Heinrich Ewald Hering in 1903[3].
At about the same time, James McKenzie used his self-invented clinical polygraph to record jugular pulse and demonstrated that the presystolic jugular "A wave" which, he stated, reflected auricular contraction, was not present in patients after they went from a normal to an irregular heart rhythm[3]. In 1901, Willem Einthoven had invented the string galvanometer for electrocardiography (ECG) for which he later received the Nobel Prize. This technology enabled Einthoven's friend and colleague, Thomas Lewis, to record and closely study the electrical activity in the hearts of patients exhibiting the pulsus irregularis perpetuus, and in 1909 Lewis' investigations led him to conclude that it was the result of what he then termed "auricular fibrillation"[4]. At the same time, Carl Julius Rothberger and Heinrich Winterberg performed similar ECG studies, and, together with Lewis, they have been acknowledged for the establishment of AF as a clinical identity[5], forming the basis for the increasing amount of knowledge on this medically challenging condition that has been accumulated during the past hundred years and a bit.

EPIDEMIOLOGY

As AF is the most prevalent cardiac arrhythmia in the world[6] and significantly increases the long-term all-cause mortality risk in both men and women[7], it is not surprising that changes in the rhythm of the palpable pulse have caught the attention of several physicians, historically. When Lewis in his publication on the abovementioned investigations stated that the abnormal heart rhythm in question was a common condition[5], although he did not base it on large epidemiological studies, he was certainly right.

On a global level, estimately 21 million men and 13 million women were living with AF in 2010[8]. The prevalence is increasing, which can be attributed to improved ability to diagnose AF and to treat cardiac as well as noncardiac diseases[9]. The prevalence of AF increases markedly with age, and the numbers are expected to further increase significantly in the next decades along with the general increase in the age of the population and prevalence of conditions associated with AF[8]. In western countries, AF has been described as one of the most important health issues[9], and in Europe and the USA one in four middle-aged persons are expected to develop AF[8]. As many patients with AF are asymptomatic when first diagnosed, this increasingly common condition has been described as a silent epidemic[10].

PATHOPHYSIOLOGY

AF is a supraventricular tachycardia, and, as the term indicates, the condition implies rapid (tachy)[11] heart action (cardia)[12]. This results from abnormal electrical activity from an anatomical location above (supra)[13] the cardiac ventricles.

The normal cardiac rhythm is a sinus rhythm, which implies that the electrical impulse that precedes and prompts each contraction of heart, arises in the sinus node, located in the right atrium of the heart. From there, the electrical signal propagates along a certain pathway which goes through the atria, the atrioventricular node, the bundle of His, the bundle branches, and the Purkinje fibers, providing for a coordinated contraction of the atria and after that the ventricles. In a heart with AF, disorganized electrical impulses arise in other areas of the atria than the sinus node. The multifactorial etiology of AF is not completely understood. Functional and structural changes in the atria, which can in the individual cases to a greater or lesser extent be attributed to clinical and genetic risk factors (see below), facilitate a complex interaction between rapidly firing atrial ectopic foci and an abnormal atrial tissue substrate which allows for perpetuation of the arrhythmia[14,15]. The resulting helter-skelter running electrical impulses do not provide effective contractions of the atria, which instead vibrate or “fibrillate”, and furthermore they hinder regular transmission of an impulse through the atrioventricular node and into the cardiac ventricles. Thus, apart from being frequent, the ventricular contractions in patients with ongoing AF are irregular, which can readily be detected by pulse palpation, as apparently noticed early by the Yellow Emperor, and documented in the characteristic ECG changes associated with AF, as demonstrated by Lewis.

Clinical risk factors and associations

Besides age, several cardiac and non-cardiac clinical risk factors of AF have been identified, including hypertension, diabetes mellitus, myocardial infarction, heart failure, valvular heart disease, left ventricular hypertrophy, cardiac surgery, hyperthyroidism, male sex, smoking, obesity, and alcohol use[16]. AF can also be provoked by rapid atrial activation which can be seen in patients with other supraventricular tachycardias, e.g. Wolff-Parkinson-White syndrome[17]. Moreover,

inflammation has been suggested to play an important role in initiation and maintenance of AF[18].

Genetic susceptibility

Given that AF primarily occurs in older patients with one or more clinical risk factors it could be imagined that it would not have a strong genetic component. On the other hand, many older patients without any of these underlying clinical risk factors do develop AF, while others, suffering from several risk factors, maintain their sinus rhythm lifelong[19], which may suggest some heritability. Indeed, a clear familial aggregation of lone AF, i.e. AF not accompanied by typical risk factors associated with AF[20], has been demonstrated[21,22]. Genetic susceptibility can follow a monogenic, but more commonly follow a polygenic pattern[23]. Variants associated with AF have been described in genes coding for proteins involved in depolarisation and contraction of heart muscle cells and communication between cells, such as cardiac sodium channels, potassium channels, sarcoplasmatic reticulum ATPase, gap junction proteins, signaling molecules, and in genes related to inflammation[23,24].

CLASSIFICATION

Various axes of classification can be used to describe AF in the particular cases. Considering patterns of presentation, duration, and spontaneous termination of episodes, AF can be divided into “first diagnosed AF”, “paroxysmal AF”, “persistent AF”, “long-standing AF”, “long-standing persistent AF”, and “permanent AF”. Applying other criteria, AF types such as “AF secondary to structural heart disease”, “focal AF”, “polygenic AF”, “monogenic AF”, “early-onset AF”, “post-operative AF”, “AF in athletes”, “lone AF” and “silent AF” have been defined[8]. Also, AF can be divided into “non-valvular AF” and “valvular AF”, the latter referring to AF with concurrent mitral stenosis or prosthetic heart valves[25].

CONSEQUENCES

While many AF patients are asymptomatic, common symptoms of AF include palpitations, shortness of breath, lack of energy, weakness, lightheadedness, and chest pain[26]. The daily life of an AF patient can be markedly affected because of reduced physical capability[27], and, based on questionnaires, the subjective quality of life has been demonstrated to be impaired[28].

As mentioned, apart from causing symptoms, AF increases mortality, in part attributed to undesirable interactions between AF and some of its predisposing factors. As mentioned, heart failure predisposes to AF, but, the other way around, AF can cause and worsen heart failure, which has to do with AF-induced structural cardiac remodeling and heart rate-related reduction of left ventricular ejection fraction. AF also impairs the prognosis in patients with valvular heart disease, and these two disorders can sustain each other[8]. Another consequence of the structural changes in the heart induced by AF is a tendency of return and maintenance of arrhythmia after initially time-limited episodes of AF. This self-perpetuating property of AF was first discovered in a study on goats by Wijffels et al., who introduced the

phrase “atrial fibrillation begets atrial fibrillation”[29]. The phenomenon has been linked to electrophysiological changes in the atria and pulmonary veins[30] and may involve changes in the intrinsic cardiac autonomic nervous system[31].

The abovementioned symptoms and detrimental effects of AF can be counteracted by different therapeutic heart rate and rhythm control strategies, the specific choice in the individual case depending on factors such as duration of the arrhythmia, symptoms, and comorbidity. The options for rate control include beta-blockers, calcium channel blockers, cardiac glycosides, combination therapy, and, should all else fail, amiodarone. Strategies for rhythm control include pharmacological and electrical cardioversion. Surgical procedures for elimination of electrical pathways that facilitate the arrhythmia include catheter-based ablation as well as more invasive strategies[8,17].

However, as mentioned in the preface, the starting point of this PhD project was the interest in the mechanisms of another complication of AF, considered the most important one[32], namely thromboembolism, which is a major cause of morbidity and mortality in AF patients, and which calls for a different category of therapy. This issue will be addressed in the next section, which is dedicated to thrombosis.

THROMBOSIS

Thrombosis can be regarded as a result of pathologic processes overwhelming the regulatory mechanisms of the vital physiological process termed *hemostasis*[33]. Therefore, in this section, a historical perspective on some early findings regarding thrombosis, will be followed by an introduction to the hemostatic system. Next, aspects of current knowledge on the mechanisms of thrombosis in general, and AF-related thrombosis in particular, are presented.

HISTORICAL PERSPECTIVE

“When it coagulates within the pulse the blood ceases to circulate beneficially; when the blood coagulates within the foot it causes pains and chills”

The statement above represents another quote from “The Yellow Emperor’s Inner Canon”[34] and illustrates that, as was the case with the tendency of the palpable pulse to become irregular in some patients, the ability of the blood to transform from a fluid to a semisolid phase and the potential harmful consequences was observed already in ancient times. Other prominent personages from before common era, including Hippocrates and Aristotle, were aware of this phenomenon, which they regarded as being a result of cooling[35]. Galen of Pergamon (ca. 130-210 of the common era) was the first to use the term “thrombosis” (in Greek thrombos means clot) in relation to limb swelling[36], plausibly caused by deep venous thrombosis. Much later, in the 17th century, Richard Wiseman described vein occlusion by blood clots[36] and furthermore, in a note on varicose veins, he wrote that:

"It proceedeth from the restagnation of gross Blood, which being transmitted into the Veins, either by reason of the depending of the Part, or from some other pressure upon the Vessel, or else by its own grossness, proves unapt for Circulation. Then instead of continuing its current proportionably to the other Parts, it stops in the place and coagulates."
(Richard Wiseman, 1676)[37]

In this wording, Wiseman arguably described two of the three factors that would, centuries later, constitute "Virchows triad", namely hypercoagulability and stasis[36,37]. The third component of this famous triad of factors, that theoretically underlie thrombosis, is endothelial injury[37]. Rudolph Virchow did not himself announce his own triad, but in the middle of the 19th century, based on experiments and observations mainly regarding thrombosis in the lungs and the legs, he formulated the statements, which were, about 100 years later, adapted by others in the conceptualization of Virchows triad. In a passage on the results of experiments on foreign bodies introduced into the lungs, which may have been important in forming the basis for Virchows triad, Virchow was, rather than *thrombus inititation*, in fact describing the *consequences* of a foreign body (which in vivo would be a pulmonary embolus) in the lungs:

"In all cases the blood formed more or less extensive clots around the introduced body'. '…the list of possible consequences of the obstruction could be grouped into three categories;
Phenomena due to the irritation of the vessel and its surroundings;
Phenomena due to blood coagulation;
Phenomena due to the interruption of the blood-stream"
(Rudolph Virchow, 1856)[37].

The mentioned factors causing the phenomena were however, by Virchow himself, referred to as requisite conditions for *thrombus propagation*, which is obviously an essential element in thrombus formation. Further details on the plausible origin of the concept termed Virchows triad, which still today serves as an important part of the general comprehension of the causes of thrombosis, are thoroughly covered by Bagot et al.[37]

THE HEMOSTATIC SYSTEM

The hemostatic system, which is essential for maintenance of the circulatory system in humans, has evolved over millions of years, and provides for the arrest of bleeding by sealing defects and facilitating repair at sites of vascular injury[38], which will occur many times during an individual's lifetime[39]. The mechanisms by which hemostasis provides for the continuation of normal blood flow in the circulation involve complex interactions between cells and many extracellular components. As the cell type that constitutes the interior surface of blood vessels, endothelial cells play a major role in hemostasis, while leukocytes also take an active share, as hemostasis and

inflammation are to a great extent interwoven processes[40,41]. Platelets, which are circulating anuclear cell fragments derived from megakaryocytes in the bone marrow, are also essential for the hemostatic process. Endothelial cells, platelets, and leukocytes are all capable of releasing extracellular vesicles (EVs), which, owing to their potential procoagulant effect, may play an important role in thrombotic and hemostatic processes[42]. While EVs will be covered more detailedly in the next section, the mentioned cell types along with some hemostasis-related molecules are addressed in the following. Two important procoagulant proteins, namely TF and von Willebrand factor (vWF), which were among the objects of investigation in study 4 of this PhD project, have been given their own subheadings. TF is also known as coagulation factor III (FIII) and thus represents one of the 13 coagulation factors involved in the coagulation cascade that leads to formation of a fibrin clot which is an essential element of the hemostasis process.

Endothelial cells

Under normal circumstances, the endothelium serves as an anticoagulant surface that lets the blood maintain its fluidity and as a barrier that separates the blood from the deeper layers of the vessel wall in which procoagulant agents such as collagen and TF are located[38,43]. The anticoagulant features of normal, intact endothelial cells include their secretion of nitric oxide (NO) and prostacyclin, which are platelet inhibitors and vasodilators, and the ectonucleotidase termed Cluster of Differentiation 39 (CD39), which metabolizes the platelet agonist adenosine diphosphate (ADP). Furthermore, endothelial cells express the anticoagulants tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), endothelial cell protein C receptor (EPCR), heparin-like proteoglycans[44], and plasminogen activators[45]. Endothelial cells are negatively charged and therefore possibly repel the likewise negatively charged platelets[38].

While the endothelium possesses many anticoagulant properties, it also has procoagulant features. Endothelial cells contain Weibel-Palade bodies, which are secretory organelles containing several proteins that enable endothelial cells to influence hemostasis and inflammation, including vWF, P-selectin[46], and E-selectin[38], the two last-mentioned mediating leukocyte and platelet rolling on endothelial cells prior to actual adhesion. The contents of the Weibel-Palade bodies can come into play in case of vascular insult or stimulation of endothelial cells by vasoactive agents such as thrombin (activated coagulation factor II (FIIa)), histamine, and bradykinin. Other factors that can stimulate endothelial cells to deviate from their nonthrombogenic protective behaviour, are hypoxia, fluid shear stress, oxidants, endotoxin, and inflammatory cytokines. It has been described that activated endothelial cells can synthesize TF and plasminogen activator inhibitor-1 (PAI-1)[38].

Leukocytes

Like endothelial cells, quiescent leukocytes facilitate the maintenance of blood fluidity, e.g. by monocyte expression of anticoagulant factors such as EPCR, TM, and TFPI. However, exposed to certain stimuli, leukocytes can undergo transformation and release procoagulant mediators. One of the effects of leukocyte-released

cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 1b is downregulation of the expression of EPCR and TM. Monocytes represent the largest intravascular source of TF, which may also to a lesser extent be expressed by neutrophils and eosinophils[47]. Neutrophils also contain and release serine proteases that contribute to coagulation by activation of coagulation factor V, VIII, and X and degradation of the anticoagulant cofactors antithrombin, heparin cofactor II, and TFPI[47]. Another procoagulant feature of neutrophils is their ability to release neutrophil extracellular traps (NETs), and while there is little evidence that NETs play a significant role in normal hemostasis, they have been associated with arterial as well as venous thrombosis[48], and will be further described under the heading "Mechanisms of thrombosis".

Platelets

In a healthy adult each litre of blood typically contains between 150 and 400 billions of circulating platelets[49]. When flowing through normal, intact blood vessels, platelets are exposed to inhibitory signaling mechanisms from the endothelial cells which allow them to remain inactivated, not adhering to the vessel walls[50]. However, on their surface, platelets express multiple specific receptors such as Glycoprotein (GP) VI which can bind collagen, GPIbα which can bind vWF, P2Y12 which can bind ADP[51], and GPIIb/IIIa which is a fibrinogen receptor, enabling the platelets to become quickly activated and exert their hemostatic functions upon vascular injury[52]. Platelets contain secretory granules, the most abundant type being the α-granule, which contains several types of proteins that play a role in inflammatory and hemostatic processes, including vWF, fibrinogen, and P-selectin[53]. Also, small amounts of TF have been identified in α-granules in platelets, potentially derived from monocyte-derived EVs incorporated after interaction with the platelet membrane via adhesion molecules[54], while de novo synthesis of TF by platelets upon activation has also been described[55]. Platelets also contain so-called dense granules that contain ADP, adenosine triphosphate (ATP), and serotonin, which can along with thromboxane A_2 (TXA_2) be released from platelets and result in activation of other platelets[56].

Tissue factor

TF, which has been established as the principal initiator of blood clotting in vivo[57], is a glycoprotein which in its full-length form (flTF) is composed of 263 amino acids and exists in various cells throughout the body, including the adventitial cells that cover all blood vessels larger than capillaries and many types of epithelial cells that surround organs[38]. Thus being omnipresent in the deeper layers of the vasculature this crucial component of the blood coagulation system has been described as a "hemostatic envelope"[58]. When subendothelial TF is exposed to blood, it unites with blood coagulation factor VII in its inactive (FVII) and active (FVIIa) form thus forming the TF:FVIIa complex which is the most potent known activator of the coagulation cascade[38] and this TF pathway has been described as the 'prima ballerina' in the initiation of coagulation[57,59]. In some cell types, such as fibroblasts, pericytes, and epithelial cells, TF is expressed constitutively, while in some vascular cells such as

smooth muscle cells and endothelial cells, which in their quiescent state do not express significant amounts of TF, expression of TF can reportedly be induced e.g. by inflammatory mediators[38,54]. The cell-associated flTF comprises a 21 amino acids long intracellular domain, a 23 amino acids long transmembrane domain, and a 219 amino acids long extracellular domain. More recently, it was discovered that also a 206 amino acids long isoform of TF, which is not membrane-integrated, exists[60]. This so-called alternatively spliced TF (asTF) is soluble in blood plasma and represents a type of blood-borne TF. Also flTF exists in a blood-borne form. TF can be expressed by monocytes, but circulating flTF has also been reported to be present in the membrane of EVs released from endothelial cells, smooth muscle cells, and platelets[54]. However, controversy exists regarding the range of cell types that are in fact able to release TF-bearing EVs[61], which will be brought up again in the section on EVs.

von Willebrand factor

vWF is a glycoprotein synthesized in megakaryocytes and endothelial cells. In its monomeric form vWF contains 2050 amino acids, and through intracellular processing some of these monomers are assembled into large hemostatically active polymeric forms, which are stored in the Weibel-Palade bodies of endothelial cells and in the α-granules of platelets[62]. vWF is capable of binding to several types of collagen and other connective tissue components[62], to which it will be exposed in case of vascular injury. As mentioned, vWF also has the ability to bind to GPIbα, but also other platelet receptors may be involved in vWF-mediated platelet recruitment to the injured part of the vessel wall[63], which is an essential element of hemostasis. Moreover, vWF is a carrier protein for coagulation factor VIII (FVIII), and high levels of vWF induce high levels of FVIII[63]. The resting endothelial cells are believed to release some vWF constitutively, while larger amounts can be quickly released by exocytosis of Weibel-Palade bodies as a response to endothelial stimulation[64]. Such stimulated vWF release from endothelial cells can be induced by various activators, including hypoxia, trauma, shear stress, thrombin, fibrin, histamine, ADP, complement, and vascular endothelial growth factor (VEGF), while it can be inhibited by NO and hydrogen peroxide[65]. vWF secretion from platelets is predominantly or entirely stimulus-induced[64].

Platelet plug and fibrin clot formation

When a blood vessel is injured, it constricts and thus limits the amount of blood that is shed from the circulation[38]. The amount of blood that does leak out comes into contact with subendothelial structures, which leads to a complex series of molecular events that provides for cessation of bleeding and repair of the vessel wall.

As mentioned above, two essential elements of hemostasis are recruitment of platelets to the site of vessel wall injury and the course of the coagulation cascade. These two elements represent each their respective component of the traditional comprehension of hemostasis, in which the process is divided into *primary* and *secondary hemostasis*, although these two subprocesses, which take place simultaneously, are highly mechanistically intertwined[43].

Platelet plug formation

The many receptors that platelets carry on their surface, allow them to respond to the vascular injury, which leads to rapid platelet activation, secretion of platelet granule contents, platelet adherence to the subendothelial matrix, platelet aggregation, and eventually platelet plug formation that helps stop the bleeding[49]. The platelet activation comes about by several mechanisms, including platelet interaction with extracellular matrix components. Activated platelets themselves provide for the activation of other nearby platelets by release ADP, ATP, serotonin, and TXA_2. Moreover, thrombin is a potent platelet activator[56], which is one example of the interplay between the so-called primary and secondary hemostasis. Another example is that activated platelets expose phosphatidylserine (PS) on their phospholipid membrane surface, which facilitates propagation and enhancement of coagulation reactions[66]. The adhesion of platelets to the site of injury relies in part on receptor-mediated interaction of platelets with collagen directly. However, vWF, circulating in blood plasma, and additionally released from Weibel-Palade bodies of damaged endothelial cells and α-granules of activated platelets play an essential role in both platelet adhesion and platelet aggregation based on its ability to bind to subendothelial matrix proteins and cell membranes as well as to receptors on activated platelets[56]. Apart from forming a plug and supporting the coagulation cascade, activated platelets are capable of recruiting leukocytes, e.g. neutrophils, whose αMβ2 and P-selectin glycoprotein-1 (PSGL-1) interact with the platelets' GPIbα and P-selectin receptors, respectively[49], while also monocytes, dendritic cells, and lymphocytes can be recruited by vessel wall adherent platelets[67].

Antiplatelet therapy

Drugs that target the activation and aggregation of platelets can be used in the prevention and treatment of some types of thrombosis. Aspirin is a cyclooxygenase inhibitor and thus obstructs the formation of TXA_2. P2Y12 receptor antagonists and glycoprotein IIb/IIIa inhibitors are other important antiplatelet drugs[43,68].

Fibrin clot formation

The term 'coagulation cascade' refers to the similarity of the common model for coagulation, which was launched in 1964[69,70], with a waterfall or a cascade. It comprises a sequence of coagulation factors in the form of proenzymes, getting converted to their active enzyme forms by the 'upstream' activated coagulation factor[56]. This chain reaction can be activated by two different pathways, termed the *intrinsic* and the *extrinsic pathway*, respectively, both of them resulting in activation of coagulation factor X (FX) and thus of the *final common pathway*, eventually leading to fibrin formation[71]. All the components needed for initiation of the intrinsic pathway are present in the blood, while initiation of the extrinsic pathway is dependent on an external factor, namely TF[56], although as mentioned, it is now known that some amount of TF actually circulates in the blood.

The extrinsic pathway, which is the more important in order to trigger the hemostatic response in vivo[38,56], is initiated with the aforementioned TF:FVIIa complex, also known as the *extrinsic tenase complex*, which cleaves FX to FXa.

Furthermore, the TF:FVIIa complex is capable of a converting FIX into FIXa[71].
The intrinsic pathway is activated when coagulation factor XII (FXII) in the blood is exposed to a hydrophilic surface to which it can bind, leading to accumulation and autoactivation of FXII, which can happen in vitro or in vivo[56,71]. Activated FXII (FXIIa) cleaves prekallikrein into kallikrein and coagulation factor XI (FXI) into activated FXI (FXIa). FXIa in turn activates coagulation factor IX (FIX) in a reaction that depends on the presence of ionized calcium, and next, activated FIX (FIXa) in concert with its cofactor, FVIIIa (activated coagulation factor VIII), forms the *intrinsic tenase complex*[38,71]. When assembled on a negatively charged phospholipid surface, such as the membrane of activated endothelial cells and platelets, the tenase complex converts FX to its activated form (FXa).
The final common pathway begins with the activation of FX by either the intrinsic or the extrinsic pathway. FXa forms part of the so-called *prothrombinase complex*, which also includes activated coagulation factor V (FVa) and is dependent on calcium and a negatively charged phospholipid surface. The prothrombinase complex converts prothrombin to thrombin, and next, thrombin cleaves fibrinopeptides from fibrinogen, generating fibrin monomers which subsequently polymerize, forming the fibrin clot[38].

The fibrin clot helps stabilize the platelet plug, and also circulating erythrocytes and leukocytes become incorporated in the structure[72]. The fibrin clot is strengthened by the thrombin-mediated activation of coagulation factor XIII (FXIII) to its active form, FXIIIa, which induces covalent crosslinking between neighbouring fibrin polymers. Besides activation of FXIII and platelets, thrombin has several functions, including activation of FV, FVIII, FXI, thereby accelerating the production of thrombin itself[56]. Thus, a positive feedback loop leads to amplification and propagation of coagulation on the surface of activated platelets[43].

Anticoagulant therapy
FII, FVII, FIX, and FX are vitamin K-dependent, meaning that their synthesis in the liver is dependent on sufficient amounts of active vitamin K. The so-called vitamin K antagonists (VKAs) which reduce the levels of active vitamin K are therefore effective inhibitors of the coagulation cascade and represent the classical type of oral anticoagulants. The more recently developed direct oral anticoagulants (DOACs) directly inhibit either FIIa or FXa[73]. Unfractionated and fractionated heparins represent another group of drugs that inhibit the coagulation cascade, which they do by markedly increasing the anticoagulant potency of one of the natural coagulation inhibitors, namely antithrombin[68].

Natural coagulation inhibition

As indicated in the description of endothelial cells and leukocytes, several mechanisms that inhibit platelet function and the coagulation cascade exist. This helps maintain proper hemostasis, which represents a balance between procoagulant systems and anticoagulant systems[43]. While thrombin is a key player in the positive feedback loop that enhances the hemostatic process, it also plays an

important role in the downregulation of the coagulation process, as it binds to TM and activates an enzyme called protein C, which is bound to the membrane protein called EPCR. Activated protein C forms a complex with its cofactor, protein S, and this complex inactivates FVa and FVIIIa and thereby inhibits the intrinsic tenase complex and the prothrombinase complex[56]. Antithrombin downregulates the clotting cascade by inhibiting thrombin and FXa and to a lesser exent by inactivating FIXa, FXIa, and XIIa[74]. TFPI is another coagulation protein inhibitor. TFPI is partially dependent on protein S and can inhibit FXa and act to inactivate the TF/FVIIa complex. Yet other modulators of coagulation exist in the fairly complex hemostatic system in which some agents have up- as well as downregulating effects on coagulation[56].

Fibrinolysis

The fibrinolytic system is also an important element in the hemostatic balance as it provides for dissolution of blood clots formed during wound healing. The enzyme named plasmin is essential in fibrinolysis. The proenzyme, plasminogen, binds to the surface of the fibrin clot or a cell surface and can be converted into plasmin by tissue plasminogen activator (tPA) or urokinase type plasminogen activator (uPA). Plasmin cleaves fibrin and thus dissolves the clot[43] Plasmin may also modulate the formation of the primary hemostatic plug, since GPIIb/IIIa and GPIbα are also plasmin substrates, and, furthermore, it may have the capacity to inactivate FVIIIa[72]. Fibrinolysis is, like the coagulation cascade, regulated by a number of inhibitors, including PAI-1, plasminogen activator inhibitor-2 (PAI-2), α2-antiplasmin, and thrombin activated fibrinolysis inhibitor (TAFI)[43,72].

MECHANISMS OF THROMBOSIS

A successful interplay between the above outlined excitatory and inhibitory influences on plug and clot formation keeps the hemostatic balance. Changes or abnormalities of the different components may tip that balance towards bleeding or thrombosis.

According to the common perception of Virchow's triad, the circumstances that on a general level predispose to thrombosis are abnormalities in the vessel wall, abnormalities in the blood flow, and abnormalities in the blood constituents[75].

Red and white thrombi

Traditionally, thrombi have been divided into so-called 'red thrombi', which are rich in fibrin and erythrocytes, and 'white thrombi', which are rich in platelets[75]. The white thrombi primarily form in the arterial system, while the formation of red thrombi takes place in low pressure systems, including veins and the cardiac atria[76]. Important factors contributing to the development of arterial thrombi include endothelial dysfunction and damage related to atherosclerosis, inflammation, hypertension, turbulent blood flow at stenotic regions and platelet hyperactivity[77].

The occurrence of venous thromboembolism (VTE), which comprises deep venous thrombosis (DVT) and pulmonary embolism (PE), is believed to be contingent on a

combination of stasis and hypercoagulability more than endothelial damage[78]. Well-established inherited risk factors for VTE include deficiency in one of the natural coagulation inhibitors protein C, protein S, or antithrombin and gain-of-function mutations in the FII or FV gene[78,79].

The classification of thrombi into predominantly platelet-mediated white thrombi developing in arteries and fibrin-rich red thrombi developing in veins is consistent with the prevailing pharmacological management of thrombosis. Antiplatelet agents, such as aspirin, glycoprotein IIb/IIIa inhibitors, and P2Y12 antagonists are the main therapeutics against arterial thrombi, while agents directed against the coagulation pathway such as VKAs and DOACs are mainly used against venous thrombi[43]. However, platelets do also play a role in the formation of venous thrombi, and antiplatelet drugs are to some extent capable of preventing venous thrombi[80]. Likewise, anticoagulant drugs are effective for prevention and treatment of some types of arterial thrombi, e.g. coronary artery disease and arterial thromboembolism related to AF[77,80]. A clear-cut distinction between red and white thrombi has also been challenged by the suggestion of epidemiological associations between venous thromboembolism and atherothrombosis which may not least be due to the fact that the two disease entities have some risk factors in common, such as age, obesity, cigarette smoking, and metabolic syndrome[81].

Thrombosis in atrial fibrillation

Thromboembolism is considered the most important complication of AF[32]. AF increases the overall stroke risk about five-fold[82] and is the most common factor contributing to stroke in the elderly[32]. Thrombogenesis in the left cardiac atrium (LA) in patients with AF is multifactorial and the mechanisms, while not fully understood, involve all three elements of Virchow's triad[83].

Stasis in the left atrium and the left atrial appendage

As mentioned in the section on AF, the fibrillating atria do not contract properly. The faulty contractile function of the left atrium (LA), along with resulting LA enlargement, leads to stasis of blood in the left atrium, which is regarded as an important factor but not the only explanation of the formation of thrombi in the LA[83]. The left and the right cardiac atrium each have an extension in the form of a muscular pouch termed the left (LAA) and right atrial appendage (RAA), respectively. The LAA is a long, tubular, hooked structure into which there is a narrow entrance from the main part of the left atrium[84]. While the LAA is believed to have a hemodynamic function by acting as a decompression chamber in periods of high left atrial pressure due to its profound distensibility[84], it is also the major site of intra-atrial thrombus formation in patients with atrial fibrillation as well as in patients with sinus rhythm[83]. In patients with AF the dimensions of the LAA change[83]. By use of transesophageal echocardiography, AF has been shown to reduce blood flow velocity and induce an abnormal flow pattern in the LAA[85], and atrial thrombus formation has been correlated with reduced LAA flow velocities[86].

Changes in blood composition

Potential associations between AF and blood plasma markers of platelet activation, coagulation activation, fibrinolysis, inflammation, and endothelial function have been investigated in various studies. A meta-analysis reported on elevated levels of the coagulation activation markers D-Dimer, thrombin-antithrombin complex (TAT), prothrombin fragment 1 and 2 (F1+2), fibrinogen, and antithrombin-III (AT-III) in AF patients[87]. In the same study significantly higher levels of the platelet activation markers platelet factor-4, β-thromboglobulin, and P-selectin were described. vWF, which is a well-established marker of endothelial dysfunction and damage[83], was also elevated in AF patients[87]. In some studies the composition of blood collected from the left atrial lumen has been investigated. However, these studies have mainly focused on patients with mitral stenosis[88-95]. In one of the studies[91], it was examined if significant differences between intra-atrial and peripheral blood thrombogenicity indices, including fibrinogen and D-dimer, markers of platelet activation (P-selectin and β-tromboglobulin) and markers of endothelial dysfunction (TM and vWF), could be detected in patients with AF secondary to mitral stenosis. No significant differences were found and the conclusion of the study was that blood samples collected from a peripheral vein reflect intra-atrial indices of coagulability, platelet activation and endothelial dysfunction[91]. This conclusion, however, contrasts the findings of another study indicating elevations in intra-atrial levels of indices of coagulation activity, platelet activation, fibrinolysis, and endothelial dysfunction compared to peripheral venous levels[94]. These differences between intra-atrial and peripheral venous levels tended to be more distinct in patients with concurrent AF, which may be a result of limited spillover from the left atrium to the systemic circulation[94]. In one study blood was isolated directly from the atrial appendages[96]. This study indicated that markers of an intra-cardiac inflammatory state and markers of an extracellular matrix remodelling process in patients undergoing coronary artery bypass grafting may predict the development of post-operative AF. The intra-cardiac inflammatory state was reflected by increased peripheral hs-CRP-levels.

As mentioned, inflammation and hemostasis are highly interrelated. Peripheral venous C-reactive protein (CRP) and interleukin-6 (IL-6) are plasma indices of inflammation and have been demonstrated to be elevated in AF patients[97]. CRP as well as IL-6 can stimulate in vitro TF expression in monocytes[83]. The relation between inflammation, AF and thrombosis is complex and a subject that is not exhausted. Since inflammation appears to be able to trigger AF and AF appears to be able to establish and maintain an inflammatory environment, it is a current matter of debate to which degree AF is both a cause and consequence of inflammation[98]. Furthermore, the development of AF-related thromboembolism has been associated with inflammatory activity[99].

While hemostatic, thrombotic, and inflammatory markers have thus been associated with AF, the mechanism of this association has not been established. It is well-known that AF is not seldom complicated by other cardiovascular diseases, and whether the increased levels of the hemostatic and thrombotic markers are actually a consequence of AF itself or instead markers of coexisting cardiovascular risk factors

is unsettled[87]. However, although controversial, it has been suggested that the increase in plasma vWF levels in AF patients is independent of structural heart disease and that vWF is a cause rather than a consequence of thrombus turnover in AF[100]. Another marker of endothelial dysfunction, namely reduced concentrations of NO, has been demonstrated in AF patients and suggested to play a role in their increased risk of thrombosis[101]. Interestingly, increased peripheral venous plasma levels of TF have also been demonstrated in AF patients[102]. The TF levels correlated significantly with plasma levels of VEGF which is a potent stimulator of synthesis and expression of TF in endothelial cells[83], and the increased levels of these two proteins may be related to endothelial damage or dysfunction[102]. Hypoxia is known to upregulate VEGF, and it has been suggested that AF may cause tissue hypoxia[102].

Changes in the atrial wall

In patients with chronic AF, the prevalence of LAA endocardial thickening with fibrous and elastic tissue has been shown to be significantly higher than in patients with sinus rhythm[103]. Fibrous thickening of the atrial endocardium has been associated with a so-called "rough endocardium" with a wrinkled appearance, and areas of endothelial denudation and thrombotic aggregations have been demonstrated to be significantly more frequent in patients with AF[104]. An immunohistochemistry study demonstrated increased endothelial expression of TF and vWF in LAAs collected from patients with nonvalvular AF and recent thromboembolism when compared with LAAs collected as autopsy specimens from patients without AF who died of noncardiac events[105]. The protein overexpression was particularly marked in tissues containing inflammatory cells, especially T cells, and in areas that also contained denuded matrix of the endocardium[105]. Another immunostaining study demonstrated increased vWF in the the LAA of patients with mitral valvular disease irrespective of whether AF was present or not[106], and Goldsmith et al. noted that mitral valve disease per se causes endocardial damage and potentially thrombogenic changes in the LAA[107].

Animal models of AF have indicated reduced expression of NO in the LA as a consequence of the loss of atrial contraction. Regarding the deeper layers of the atrial wall, it has also been suggested that AF could be associated with chamber specific changes in myocardial collagen content and amounts of proteins involved in degradation of extracellular matrix and that that disruption of the extracellular matrix may be linked to thrombogenesis by introducing fibrosis and infiltration of the endocardium[83].

Thrombosis prophylaxis in atrial fibrillation

As mentioned, the cardiac atria represent low pressure environments as do veins, which, according to the distinction between red and white thrombi, is consistent with the fact that anticoagulant therapy more effectively than antiplatelet therapy lowers the risk of AF-related thrombosis[77]. An immunohistological study on the composition of thrombi present in the atrial appendage and thrombi having embolized to an artery in patients with AF indicated that fibrin and amorphous debris are the main components of atrial thrombi in situ, while in embolized thrombi the

main components are fibrin and platelets[108].
The current guidelines from the European Society of Cardiology (ESC) indicate that oral anticoagulant therapy (OAC) can prevent the majority of strokes in AF and that, except for patients at very low stroke risk, OAC should be used in most patients with AF, although bleeding risk related to anticoagulant therapy must also be considered when planning the prophylactic strategy for a given patient[8]. The recommended basis for decision is the so-called CHA_2DS_2-VASc score which takes into account important risk factors of stroke in AF patients, namely congestive heart failure, hypertension, age, diabetes mellitus, vascular disease, and sex[8]. Plasma biomarkers are not part of this risk score system, although vWF has been suggested to have a prognostic value in AF, and, in AF patients receiving anticoagulation therapy, D-dimer has been shown to predict thromboembolic events[100].
Various bleeding risk scores exist, including the so-called HAS-BLED score that takes into account hypertension, abnormal liver/renal function, stroke, bleeding history or predisposition, labile INR, age (elderly), and concomitant use of drugs predisposing to bleeding, such as platelet inhibitors or nonsteroidal anti-inflammatory drugs[8].

Recently discovered potentially prothrombotic factors

While risk of thrombosis in cardiovascular disease is predominantly evaluated by clinical factors, there is a continuous pursuit of elucidation of the complex mechanisms of thrombosis and of biochemical and cellular parameters that will allow for more refined risk assessment and more precisely targeted approaches to thrombosis prevention[109].

Postprandial changes in blood composition

Postprandial elevations of triglyceride-rich lipoproteins (TRLs), which include chylomicrons and very low density lipoproteins (VLDLs) are related to the risk of atherothrombotic disease, and it has been debated to which extent postprandial activation of the coagulation system is a contributing factor[110,111]. Increased plasma levels of FVIIa, TAT, and F1+2, and EVs in the postprandial state have been reported[110].

Blood-borne tissue factor

Motton et al. hypothesized that postprandial lipemia is associated with an increase in the levels of blood-borne TF and performed a study that did indeed indicated elevated postprandial levels of circulating TF[111]. The authors stated that the blood-borne TF which they detected using an enzyme-linked immunosorbent assay (ELISA) represented both EV-associated TF and soluble asTF. The increased levels of circulating TF were not accompanied by significant changes in TF activity[111].
In the aforementioned immunohistological study on the composition of AF-related thrombi, TF was colocalized to areas that were rich in platelets and granulocytes, and it was suggested that circulating TF may play a part in thrombus initiation as well as propagation[108].

Procoagulant extracellular vesicles

EVs, which appear to account for a greater or lesser part of total amounts of circulating TF, have been the pivot for much of the experimental work performed in this PhD project and will be further introduced in the next and final section of the background chapter.

Along with EVs, NETs have been mentioned as more recently discovered players in in thrombotic processes and potential targets for antithrombotic therapy[48], and in study 4 of this project, we took interest in their potential contribution to thrombosis in AF. An introduction of NETs will close the present section.

Neutrophil extracellular traps

The discovery of NET release as a mechanism used by neutrophils for microbial killing was made in 2004[112]. NETs are web-like structures consisting of chromatin filaments and proteins such as histones and serine proteases released by activated neutrophils as a response to microbial and inflammatory stimuli[113]. Activation of neutrophils can occur via stimulating agents such as cytokines, growth factors and the complement system[114].

It is well-described that neutrophils are an essential part of the innate immune system. They are quickly recruited to pathogen-infected tissues but also to sites with sterile injury, extravasating from the blood and entering affected tissues where they phagocytically engulf microorganisms and cellular debris. The release of NETs, so-called 'NETosis' which can take place intravascularly, represents another protective mechanism performed by neutrophils. The chromatin structures which, when released, are markedly larger than the neutrophils themselves, are capable of immobilizing pathogens, restraining them in an area with a high concentration of antimicrobial proteins such as neutrophil elastase and myeloperoxidase released from the neutrophils' intracellular granules[115,116]. NETs represent one of the many links between inflammation and coagulation. NETs may play a role in so-called immunothrombosis which represents a form of protective thrombosis taking place in microvessels, limiting the spreading and survival of circulating microbes. However, apart from the protective functions, NETosis can also promote harmful thrombosis. In animal models NETs have been shown to enhance in vivo venous thrombosis, and extracellular nucleosomes, which constitute the main part of NETs, have been shown to promote arterial thrombosis[116].

Different mechanisms link NETosis and coagulation. Neutrophil elastase can cleave and inactivate TFPI and thus enhance procoagulant activity which leads to platelet activation, and activated platelets can in turn enhance NET formation[117]. Neutrophils and platelets both release EVs that may express TF and may be trapped by NETs[116]. Furthermore, NETs can trigger the intrinsic coagulation pathway via FXII activation and may inhibit tPA-mediated fibrinolysis[116].

While NETosis was initially regarded as a process solely occurring as part of a cell-death program during which the NET-releasing neutrophil would perish, it has more recently been discovered that also another form of NETosis, termed vital NETosis, which takes place more rapidly and which allows the neutrophil to survive and

maintain its phagocytic function, occurs[113,114]. While in suicidal NETosis the chromatin and proteins are released from the neutrophil via disruption of the plasma membrane, the release mechanism in vital NETosis involves transport of the NETs to the plasma membrane by intracellular vesicles whose contents are then released into the extracellular space[114].

EXTRACELLULAR VESICLES

EARLY EVIDENCE

"When clotting occurs, coagulant particulate material is released from platelets, considerably in excess of what is required for thrombin generation and it can be detected in serum. Its presence accounts for the platelet-like activity of serum."

The quote is from the scientific paper "The Nature and Significance of Platelet Products in Human Plasma" published by Peter Wolf in 1967. Wolf described this particulate material, which he termed "platelet dust", as sedimentable by high-speed centrifugation, visualizable by electron microscopy, and rich in lipid content[118]. The characterization of platelet dust is regarded as a milestone in the field of EVs[42,119,120]. When the initial discovery of EVs is discussed, Wolf's work is often mentioned in connection with another paper published two decades earlier, namely "Biological Significance of the Thromboplastic Protein of Blood" by Erwin Chargaff and Randolph West[121]. Chargaff and West demonstrated that after a 150 minute high-speed centrifugation of blood at 31,000 x g the supernatant plasma lost coagulation capacity as indicated by increased clotting time, while

"The particulate fraction sedimented at 31,000g probably includes, in addition to the thromboplastic agent, a variety of minute breakdown products of the blood corpuscles."
Erwin Chargaff and Randolph West, 1946[121].

As it appears from these early descriptions of a sedimentable blood plasma fraction containing particles released from cellular elements and exhibiting coagulant capacity, the cradle of EV research was anchored to an interest in the mechanisms of blood coagulation and, in a chronological perspective, it rocked alongside the studies that formed the basis for the coagulation cascade model.
Since then, the field of EVs has evolved markedly and has attracted growing interest from researchers in hemostasis and thrombosis but also in several other branches of medical and non-medical science.

CURRENT STATE OF THE ART

In recent decades an increasing amount of evidence has indicated that several types of cells release vesicles of varying size into various body fluids by different mechanisms and that these vesicles are involved in numerous physiological and

pathological processes[122]. Apart from blood, EVs have been isolated from cerebrospinal fluid, amniotic fluid, milk, saliva, nasal secretions, ascites, pleural effusions, bronchoalveolar lavage, bile, synovial fluid, ocular effluent, aqueous humor, semen, and urine[123]. The cellular origin, molecular content, functional properties, and amounts of EVs depend on the type of body fluid and have been demonstrated to be associated with bodily conditions and diseases such as inflammation, cardiovascular disease, and cancer[124]. EVs can contain many of the same membranous and cytosolic elements as their parent cells and exert their biological effects in different ways. A key feature of EVs seems to be their ability to transfer information from one cell to another. In this way they represent a cell-to-cell signaling method which supplements the more classically described juxtacrine, autocrine, and endocrine signaling forms that are continuously brought into play in humans and other multicellular organisms. The EV-associated signals can be transmitted by molecules such as proteins, carbohydrates, and lipids[42]. Studies from the past ten years have shown that the 'cargo' of EVs can also include some deoxyribonucleic acids (DNAs) and several subtypes of ribonucleic acids (RNAs), which can be transferred via the EVs to recipient cells[125]. The transport of molecules by EVs does not only function as a means of intercellular communication but also as a way to eliminate molecules, e.g. amyloid proteins and modified RNAs, from cells[122].

Along with the awareness of their existence and potential biological significance, an interest in the potential use of EV measurements for diagnostic and prognostic purposes has developed. Also, EVs have been mentioned as potential future therapeutic targets[48,126,127]. However, EVs are a very heterogenous group of analytes, and it is challenging to isolate, quantify, and characterize them in order to distinguish the different types of EVs from each other and from similarly sized components in their environment.

Nomenclature and mechanisms of release

The complexity and heterogeneity of these cell-derived phospholipid-bilayer membrane-enclosed particles, that can collectively be termed EVs, reflect in problematics of the nomenclature in the field. In some contexts, vesicles have been given names such as "prostasomes" or "synaptic vesicles" based on the cells or tissue of their origin[119], in other cases based on their biological function, e.g. "tolerasomes" for vesicles that induce immunological tolerance[128]. Broader terms have been used to designate the main types of EVs, based on the mechanisms of their biogenesis and release. In general terms, cells have been decribed to release EVs under conditions that imply 'cell stress', i.e. upon cell activation or during apoptosis[124,129]. In recent reviews on the topic, two main types of EVs released from vital cells are mentioned, namely *microvesicles* (MVs) and *exosomes*[42,122,125,130], while *apoptotic bodies* released from cells undergoing apoptosis can be regarded as a third main type of EVs[42,124,125]. These three types of EVs will be introduced in more detail after a brief description of factors that can trigger EV release.

The range of stimuli that may potentially cause cell stress or activation leading to EV release is not limpid but several pathways appear to exist. In vitro studies have indicated that release of EVs from the plasma membrane is induced by stimuli that give rise to an increase in intracellular calcium concentration, and treatment with calcium ionophores, which allows divalent cations to cross cell membranes, has been shown to increase MV and exosome release from leukocytes[131]. Bacterial lipopolysaccharide (LPS) has also been demonstrated to induce EV release from dendritic cells[132] and endothelial cells[133]. Endothelial cells also form EVs in vitro after stimulation with various cytokines and reactive oxygen species[133]. The effect of food intake on the EV population in the blood is not well known[123]. However, consumption of a high-fat meal has been shown to increase levels of endothelial EVs, and although the mechanism is not clear, endothelial EVs were suggested to be markers of endothelial dysfunction induced by postprandial hypertriglyceridemia[134]. Platelet-derived MVs are released as a response to shear stress and agitation as well as stimulation with collagen, thrombin, or ADP. Platelet activation leading to MV release can take place via their thrombin receptors, GPVI collagen receptors, or the P2X purinoreceptor 7, which is a receptor for ATP. However, a study has also demonstrated increased concentrations of platelet-derived EVs in the postprandial state, interpreted as an indication of hypertriglyceridemia-induced platelet activation[135].

Activation of B cells, T cells, mast cell, and reticulocytes via their surface receptors and subsequent EV release from these cells has also been described[133].

Furthermore, constitutive release of EVs from cells such as natural killer cells[42], T cells, and B cells[131] has been described. Moreover, tumor cells have been reported to constitutively secrete MVs, exosomes, and apoptotic bodies[133]. EV secretion may also be affected by gene polymorphisms[133].

Exosomes

Exosomes originate in the endocytic system of the cell and are subsequently secreted through the plasma membrane by a mechanism initially discovered in electron microscopy (EM)-based studies in the 1980s[136].

Endosomes are membrane-enclosed *intracellular* compartments formed during a cell's internalization of extracellular components in a process that involves invagination and fission of the plasma membrane. Endosomes contain fluid, solutes, macromolecules, particles, and plasma membrane components[137].

Inward budding of the membrane of the early endosome can take place and lead to the formation of intraluminal vesicles (ILVs) which accumulate in the endosome, resulting in the transformation of the endosome into a so-called multivesicular body (MVB). ILV formation can follow different pathways, the best characterized being the endosomal sorting complex required for transport (ESCRT) pathway, which involves four protein complexes termed ESCRT-0, -I, -II, and –III and some accessory proteins, each playing their part in binding, sorting, and clustering receptors and other proteins at the endosomal membrane and incorporating them in ILVs. A transmembrane protein superfamily termed tetraspanins is involved in the initial clustering of this ILV formation machinery, which takes place in tetraspanin-enriched

microdomains (TEMs) of the endosomal membrane. Another ILV formation pathway involves lipid rafts, which are specialized membrane microdomains with a lipid composition that differs from other regions of the endosomal membrane. Thus, sphingomyelin is clustered in lipid rafts and converted into ceramide, which in turn induces inward membrane budding so ILVs are formed[122,125,138].

In the majority of cells, most MVBs fuse with lysosomes that degrade their cargo, but some MVBs instead travel to the plasma membrane and fuse with it, releasing their content, to the extracellular compartment. After release, the former ILVs are termed exosomes. The transport of ILVs to the membrane and the release of exosomes depend on interactions between the plasma membrane and the cytoskeleton as well as local enzymatic degradation, and activation of mechanisms that facilitate membrane fusion. The tetraspanin protein superfamily is involved in the secretion as well as the biogenesis of exosomes[125]. Studies on the composition of exosomes indicate that they contain specific subsets of proteins, RNAs and lipids rather than a random selection of these molecules from their parent cell. This substantiates the perception of the existence of mechanisms for sorting specific molecules into ILVs prior to their release as exosomes although the composition varies considerably between different exosomes[131]. In relation to the description of platelets in the previous section, it can be mentioned that the α-granules in platelets also represent a type of matured MVBs that have been provided with endogenous cargo such as vWF from the Golgi apparatus of megakaryocytes[139].

In EM studies, the diameters of ILVs have been estimated to range between 30 and 100 nm[131], and the maximal diameter of exosomes were earlier described as being around 100 nm[119,136,138], while later reviews suggest a maximal exosome diameter of 150 nm[122,125,140], although at the ISEV meeting in 2015 diameters up to 250 nm were reported[141].

Microvesicles

MV biogenesis takes place at the periphery of the cell by direct outward budding from the plasma membrane in processes that on a general level mainly rely on membrane constituents, cytoskeletal functions, and proteins recruited for cutting the plasma membrane. On a more detailed level, different mechanisms have been described, and the specific mechanisms that come into play for MV biogenesis may vary between cell types and function[125].

Consensus exists that a central element in MV formation is disruption of the asymmetry of lipids in the plasma membrane. In the normal plasma membrane some phospholipid subtypes, including PS and phosphatidylethanolamine, are predominantly situated in the inner leaflet of the lipid bilayer, while phosphatidylcholine and sphingomyelin are enriched in the outer leaflet. This membrane asymmetry is influenced by lipid translocases, which exchange lipids between the internal and external leaflet. Flippase is an inward directed lipid translocase that serves to maintain membrane asymmetry by flipping divagated PS and phosphatidylethanolamine back in place in the inner leaflet, while floppase is an outward directed lipid translocase, and scramblase is a bidirectional translocase that promotes unspecific redistribution of lipids between the leaflets[142]. Flippase and

floppase use ATP when they translocate lipids, while scramblase is ATP-independent[143]. Mechanisms that lead to local increase in intracellular calcium concentration interrupt membrane asymmetry, since calcium stimulates floppase and scramblase activity while flippase activity is attenuated by elevated calcium concentration. The redistribution of PS to the external membrane leaflet is particularly important in this connection, since the thereby occurring structural imbalance makes the lipid bilayer bulge in the direction of the outer leaflet and promotes MV release. The PS translocation may potentially be sufficient for inducing MV release, but moreover, increased cytosolic calcium concentration activates intracellular proteins such as calpain and Rho kinase, which are responsible for cleaving and changing the membrane cytoskeleton, which leads to cellular contraction and membrane blebbing[120,125].

As mentioned, different mechanisms for MV release may be brought into play in different situations. Thus, changes in ceramide content in the external leaflet of the plasma membrane induced by acid sphingomyelase can also promote membrane curvature and MV release, and more recently, it was suggested that components of the ESCRT system that was mentioned in the description of exosome biogenesis may play an important role in MV biogenesis at the plasma membrane as well[122,125]. MVs contain the same overall types of cargo as exosomes, and PS is not restricted to MVs but also exists on exosomes[125].

As for the size of MVs, different ranges such as 200-500 nm[122], 100-1000 nm[136], 150-1000 nm[131], and 50-1000 nm[144] have been proposed, so looked upon as a group they have a broader size range and are generally larger than exosomes.

As mentioned, the nomenclature in the field has not been stringent, and terms as *ectosome*, *microparticle*, and *shedding vesicle* have been and are still used more or less synonymously with the term MV in the literature[42,125,131]. In this context, the term MVs is used for EVs released by direct budding of the plasma membrane of vital cells, which appears to be the preferred term in the ISEV community[42].

Apoptotic bodies

Apoptosis is programmed cell death that occurs as part of tissue homeostasis. Apoptosis can be induced via an extrinsic pathway that involves the interaction of a transmembrane receptor with a ligand, e.g. TNF-α, or via an intrinsic pathway as a response to lack of certain growth factors, hormones, or cytokines or the presence of stimuli such as toxins, hypoxia, and hyperthermia. Cells undergoing apoptosis form blebs at the plasma membrane in a process that involves caspase dependent degradation of the cytoskeleton, loss of membrane interaction with the cytoskeleton and externalization of PS to the outer leaflet of the phospholipid bilayer. Membrane blebbing leads to budding and release of apoptotic bodies containing cytoplasm, organelles, and in some cases a nuclear fragment[145]. Apoptotic bodies, also termed apoptotic vesicles[124], may contain relatively more genomic DNA than other types of EVs[122]. While apoptotic body release involves some of the same mechanisms that are brought into play in MV release, during apoptosis the PS redistribution mainly relies on a calcium independent caspase. Although the process of packing cellular debris into apoptotic bodies has not been detailedly characterized, indications exist

that it involves a highly regulated process in which particular cellular contents are incorporated in these EVs, which are released for subsequent phagocytosis, as phagocytes will perceive their PS exposure as an “eat me”-signal. In this way clearing of potentially harmful, inflammation inducing substances in membrane-enclosed entities prevents their leakage into tissues[125].

Interestingly, although MVs and exosomes also expose PS in their outer membrane leaflet they can have longer life-times and intercellular signaling functions. It has been suggested that MVs may not possess certain accompanying “find me” signals necessary for attraction of phagocytes, that PS on MVs may be less abundant and presented differently, and that a surface coating provided by proteins recruited during MV budding may prevent interactions with PS[125].

The size range of apoptotic bodies has been reported to range from diameters of 1000 nm to diameters of 5000 nm[119], although some may be down to 500 nm[146], but their size distribution has not been systematically investigated[125].

Fate and function

While apoptotic bodies are destined for phagocytosis and thus primarily represent a stage in the process of cell dismantling and biomolecule recycling[125], it has been suggested that inappropriate clearance of apoptotic bodies play an important role in systemic autoimmune disease[119].

MVs and exosomes, on the other hand, are regarded as potential mediators of advanced communication between cells and contributors to processes such as coagulation, angiogenesis, inflammation, and modulation of immune responses[124]. Thus, they appear to have destinies beyond allowing cells to dispose of waste material, and blood content of EVs can be regarded as a dynamic balance between their release and clearance[147]. Clearance of EVs from the circulation has been suggested to take place via interactions with recipient cells, uptake in the reticuloendothelial system, or excretion via the liver or kidneys[148].

The phospholipid bilayer enclosing the EVs enables them to carry and protect their internal cargo and travel to target cells on which the EVs can exert their effect via ligand-receptor-based interactions or direct delivery of cargo into the cytosol of the recipient cell after EV fusion with the plasma membrane[122,140]. The interest in and research on EVs as carriers of functional molecules between cells did not least gather speed after the discovery in 2007 of exosomes containing messenger RNAs and microRNAs and the successful in vitro transfer of functional translatable mRNA via mast cell-derived exosomes from donor cells into recipient mast cells[149]. However, although in a review article from 2017, Maas et al. mentioned that in vivo cells continuously exchange EVs over short and long distances, potentially even occasionally crossing the blood-brain barrier, in the same paper the field is described as still young and holding several ‘black holes’, one of them being the true spatiotemporal fate of EVs in vivo[122]. In another recent review Kalra et al. stated that whether packaging of cargo into exosomes, MVs, and apoptotic bodies is actually a selective or random process is largely unknown, thereby urging some caution when interpreting the ostensible cell-communication-related functions of EVs[125]. However, the omnipresence of EVs in biological fluids and more or less specific

changes in their molecular content depending on the type and pathophysiological state of the parent cells, make them interesting as potential biomarkers. Changed levels of subtypes of EVs have been identified in wide range of disorders, including metabolic, hematologic, infectious, autoimmune, cardiovascular, and neurological diseases as well as in preeclampsia, and several cancers[122,133].

In the context of this PhD project, the potential role of EVs in hemostasis and thrombosis was of particular interest. The mechanisms by which EVs expectedly affect the coagulation system do not necessarily involve sophisticated transfer of internal EV cargo to recipient cells but instead appears to be based mainly on their membrane content.

Extracellular vesicles in hemostasis and thrombosis

As mentioned in the beginning of this section, the first evidence of the existence of EVs was related to their procoagulant properties. While the potential relation of EVs to many other medical fields has been investigated, their ability to enhance coagulation is still regarded as their most widely accepted physiological role and one of their best characterized features[42,146].

Early indications on the existence of blood-borne TF and its possible association to EVs was published by Giesen et al. in 1999, questioning the previously prevailing paradigm that TF under normal conditions is not present in blood but exclusively resides outside the vascular lumen[150]. The investigators observed that when pig aortic media and collagen-coated glass slides, none of which stained positive for TF in themselves, were perfused with native human blood, thrombi staining positive for TF formed on the glass slides as well as on the aortic media. Repeating this experiment with the addition of anti-human TF antibody resulted in markedly reduced thrombus formation. Immunoelectron microscopy of the thrombi, which consisted primarily of fibrin, revealed that TF predominantly colocalized to membrane-enclosed vesicles and that these vesicles were often clustered next to platelets. Furthermore, the presence of TF-containing monocytes and neutrophils in the blood was demonstrated. Based on their results, the authors suggested that arterial thrombus propagation involves deposition of blood-borne vesicle-associated TF on platelets in the developing thrombus, allowing for coagulation factors to be in close proximity to TF and the platelet surface at the same time[150].

Since then, several experimental studies on the potential role of EVs in thrombus initation and propagation have been performed, not least by a Boston-based research group, who established a method for in vivo brightfield microscopic imaging of thrombus formation[151]. The method involved labeling of cellular and protein components in the blood using fluorescent antibodies, thus allowing for studying the pattern of accumulation of these components in a growing thrombus after prompting its formation by inducing an endothelial injury in a cremaster muscle arteriole in a living mouse. Among the findings in this in vivo model was accumulation of TF, accumulation of platelets expressing P-selectin, and generation of fibrin at the site of endothelial injury. Advanced studies, which included investigations on P-

selectin null mice and infusion of isolated monocyte-derived EVs, indicated that the accumulation of TF-bearing EVs is due to binding of P-selectin on activated platelets to PSGL-1 on the EV surface[152]. Leukocytes, another potential supplier of blood-borne TF to the site of injury, were also incorporated in the developing thrombi. However, studies on the kinetics of accumulation of TF, EVs, and leukocytes in the thrombi suggested that TF originating from hemopoietic cells is provided via EVs in the initial phase of thrombus generation while leukocytes themselves accumulate minutes later[153].

As described in the section on the hemostatic system, the tenase and prothrombinase activity that leads to downstream fibrin generation, depend on the presence of a negatively charged phospholipid surface. Such a surface exists on activated platelets and endothelial cells, and in addition on EVs. Thus, the presence of PS molecules on the external leaflet of EVs, not least MVs on which PS is often abundant, is considered to enable these vesicles to facilitate the assembly of coagulation factors and thus promote coagulation[126]. Since MVs from platelets express receptors for both collagen and vWF, it has been stated that they can be viewed as smaller versions of activated platelets[154], and an in vitro study has indicated that platelet-derived MVs actually display even considerably higher procoagulant activity than activated platelets[155]. Importantly, some EVs carry TF in their membrane, and it has been stated that MVs which expose TF and PS at the same time have the highest level of procoagulant activity[154]. While most of the evidence on TF-bearing EVs concerns MVs, studies on cancer-related EVs have indicated that exosomes can also carry TF[156].
The abundance, origin, and biological relevance of TF-exposing EVs in the blood in physiological and pathological contexts is not clear. While studies have indicated that platelets, or megakaryocytes, are the major source of PS-bearing MVs, monocytes have been stated to plausibly be the main source of TF-bearing MVs. TF-bearing MVs may under some conditions be released by other cell types, including neutrophils, endothelial cells, and platelets, although contradictory data exist on this topic[154]. An in vitro study by Aleman et al. indicated that while MVs from both leukocytes and platelets can contribute to clot *propagation*, the leukocyte-derived MVs were capable of also *initiating* the 'extrinsic' TF-dependent pathway. The platelet-derived MVs did not exhibit such TF activity, but nevertheless they accelerated thrombin generation and fibrin formation when mixed with the monocyte-derived ones[61]. Sabatier et al. demonstrated that endothelial cell-derived MVs can interact with monocyte-derived MVs in vitro and make them exhibit increased levels of TF antigen and TF mRNA as well as increased procoagulant activity[157], while the direct contribution of endothelial EVs to blood-borne TF has been described as probably limited in the absence of stimulation but potentially important in case of drastic endothelial activation[126].
Hoffman et al. conducted an in vitro study in a mouse model, which suggested that blood-borne TF can contribute to thrombosis in some circumstances but probably not to normal hemostasis[158].
Some recent studies have suggested that MVs may also promote coagulation via

mechanisms that are independent of TF and the extrinsic pathway but instead involve FXI and FXII, although additional investigations are needed to gain thorough insight into this aspect[146]. At present, the major determinant of the coagulant capacity of EVs is believed to be the presence or absence of TF in their membrane[42], while considerable procoagulant potiential is also ascribed to PS[147]. It should also be noted that MVs do not only exhibit procoagulant capacity but also have been demonstrated to possess the ability to *down*regulate coagulation by harboring of TFPI on their membrane, supporting protein C and protein S mediated regulation of coagulation, and assisting plasmin generation. These findings have given rise to the view that the complex role of MVs in coagulation represents a balance between pro- and anticoagulant properties and that their net effect in hemostasis and thrombosis is at any time determined by the current state of that balance[146].

The interplay between different cell types and the EVs released from them in relation to hemostasis and thrombosis appears complex and many-faceted, and many sorts of interactions such as fusions and exchanges between the membranes of EVs from monocytes, endothelial cells and platelets may take place in vivo and in vitro[126]. Furthermore, when aiming to obtain preparations of EVs with specific cellular origin some degree of contamination from other EV subtypes must be expected[61], which represents one of the experimental challenges associated with this field.

Whether or not signicant levels of functional blood-borne TF exists in the blood of healthy humans has been debated. Conflicting results have been reported, and some investigators have found very low levels of EV-associated TF activity[154], which may be undetectable by some methods[147]. Different posttranslational modifications, such as glycosylation, of TF may result in different versions of TF exhibiting different levels of procoagulant activity, and it has been suggested that EV-associated TF may normally be present in a so-called 'encrypted' low-activity state, so that it will not activate the coagulation cascade untimely. The encrypted TF may then, when timely, be activated by different mechanisms, including potential conformational changes induced by PS[154].

In a review from 2016, Mooberry et al. noted that increased levels of circulating MVs have been reported in many inherently prothrombotic conditions, including malignancy, sepsis, thrombophilia, trauma, nephrotic syndrome, inflammatory bowel disease, systemic lupus erythematosus, pregnancy, and preeclampsia[146]. However, the authors stressed that in spite of these associations and in some cases also a correlation between MV concentrations and thrombotic events, a causal relationship between the increased MV levels and thrombotic events does not necessarily exist. They also noted that only few prospective data exist and that these few data display conflicting results, possibly due to differences in applied methodologies. Anyhow, one prospective study found TF-positive MV levels measured by flow cytometry (FC) predictive of thrombosis in brain tumor patients

while another prospective but small study indicated that increasing levels of MV associated procoagulant activity over time correlated with subsequent development of thrombosis[146].

Extracellular vesicles in atrial fibrillation

AF was not on the abovementioned list of prothrombotic conditions with increased circulating EV levels, and only a few studies have investigated EV levels in AF patients. In 2005, Chirinos et al. published a study on the potential effect of Digoxin use on peripheral venous plasma levels of markers of platelet and endothelial cell activation in patients with nonvalvular AF[159]. Increased levels of endothelial-derived MVs were detected in the Digoxin users, whereas no differences in platelet-derived MVs were found after adjustment for potential confounders.
In a study published in 2007, Choudhury et al. hypothesized that the peripheral venous plasma concentration of platelet-derived EVs are elevated in nonvalvular AF patients as compared to healthy control subjects and furthermore as compared to a group of disease control subjects with a history of hypertension, coronary artery disease, diabetes, or stroke[160]. While data showed no difference in platelet-derived EV levels between AF patients and disease control subjects, both AF patients and disease control subjects displayed significantly higher levels than the healthy control subjects. AF did not prove to be an independent determinant of the concentration of neither platelet-derived EV levels nor soluble P-selectin, and the authors suggested that platelet activation in AF patients results from underlying cardiovascular disease.
In another paper from 2007, Ederhy et al. found increased plasma levels of PS positive EVs in AF patients as compared to a control group without cardiovascular risk factors and compared to a control group with cardiovascular risk factors[161]. Regarding subgroups of EVs, i.e. endothelial-derived and platelet-derived EVs, the AF group and the control group with cardiovascular risk factors had similar levels, which were higher than in the group without cardiovascular risk factors.
In 2009, Azzam et al. demonstrated that patients with valvular AF had higher plasma levels of platelet-derived EVs than healthy control subjects and that the severity of mitral stenosis correlated with levels of platelet-derived EVs[162].

In three of the abovementioned four studies on EV levels in AF patients, EV measurements were performed with FC, which reportedly is the most commonly used approach to EV analysis[146]. While FC can at high speed provide phenotypic information on EVs in a sample, the method also has considerable limitations as do other methods for EV detection, which along with preanalytical variables makes EV analysis on blood samples a challenging discipline.

In the following, methods for EV detection are presented, and subsequently preanalytical and analytical issues, which can influence the results on EV measurement in blood, are introduced. Since the interference of lipoproteins on EV

measurements has been a focal point in this PhD project, space is given to an introduction of this group of particles.

ANALYSIS

Characterization of the EV population in a body fluid may include determination of variables such as the concentration, size distribution, morphology, biomolecular cargo, and membrane content. Depending on the features of interest, different methods can be applied, and combination of techniques may be relevant[141].
Microscopy techniques such as electron microscopy, as applied by Wolf[118] and Giesen[150] in their path-breaking work in the 1960s and 1990s, respectively, and atomic force microscopy can be used for studying size and morphology. Also, by use of antibody-based labeling, biochemical information can be obtained with these types of microscopy, while they are not suited for concentration determination[163].
Several other methodologies, including classical immunoassays, have been used for EV analysis and each has its advantages and disadvantages[146]. Western blotting can be used for determination of the proteins present in a pellet containing EVs but not for determination of EV concentration or size. ELISAs can capture some of the vesicles present but is not suited for discrimination between EV subtypes and also detects soluble antigens[164]. Specific clotting activity assays in which the measured clotting time is dependent on the amounts of procoagulant phospholipids (PPLs) have been used for estimation of levels of PPL-exposing EVs[165].
A number of optical, light scatter-based methods have been applied[163], including FC, and nanoparticle tracking analysis (NTA), which will be briefly introduced in the following along with two other more recently launched methods for EV detection that have been explored and used in the studies done in this PhD project, namely tunable resistive pulse sensing (TRPS) and Extracellular vesicle Array (EV Array).

Flow cytometry

FC has been widely used for blood cell counting and characterization for decades and is currently also a common method for EV analysis which enables detection of tens of thousands of EVs per minute[141]. In a flow cytometer, particles are transported in a fluid stream through a detection device that detects the particles based on measurements of their impedance, fluorescence, and/or scattering of laser light applied in the flow cytometer. FC used for EV analysis relies on detection of light scattering and fluorescence, while impedance is measured in the more recently developed technology termed tunable resisistive pulse sensing (TRPS).
When a particle passes through a laser beam, it scatters the light in different directions, depending on properties of the particle. The scattered light is measured by different detectors that can detect forward (FSC), and side scattered light (SSC). Theoretically, particles larger than the wavelength of the laser light will predominantly scatter light in a forward direction, and therefore FSC signal is associated with particle size[163] and can be used to estimate the particle diameter. Particles smaller than the wavelength of the laser light will scatter relatively more light in a perpendicular direction[163]. When FC is a applied on a medium containing

cells, SSC is highly influenced by the internal composition of the cell, including granularity and nucleocytoplasmatic ratio[166]. When FC is used for analysis of EVs, which are much smaller than cells, SSC from the surface of the EVs tends to overwhelm SSC from the structures inside the vesicles[163]. However, the SSC signal can allow for a better resolution of small particles than when FSC is used alone[166,167]. Conventional FC does not allow for detection of particles smaller than around 300 nm[164,168] and since the majority of EVs are below this size it has been stated that FC is only capable of identifying the 'tip of the iceberg'[169,170] of EVs in a biological sample. Recently, new flow cytometers have been developed, allowing for flow cytometric detection of polystyrene particles as small as about 100 nm[141] but the light scatter intensities of the smallest EVs may often be below background noise[171]. The sensitivity and specificity of FC can be further increased by fluorescent labeling of the EVs[141]. Labeling EVs can help distinguish EVs from other particles present in the studied medium, and cell type specific antigens in the EV membrane can serve as markers of cellular origin[167].

While the principles of FC are well suited to detect, count, and characterize EVs, further instrument improvements are needed for full EV phenotyping in biofluids[171]. At meetings in ISEV and the International Society on Advancement of Cytometry (ISAC) it was agreed that other measurement techniques such as NTA and TRPS should be used alongside FC[141].

Nanoparticle tracking analysis

Like FC, NTA is based on detection of scattered light but the principle of the method is very different. In NTA, which was introduced in 2006, the suspension of interest is illuminated by a laser beam via a glass prism. Particles in the suspension scatter light, and a microscope with a video camera collects the scattered light in the field of view, thus visualizing and recording each particle present in the field of view. The recorded video is analyzed by NTA software, which provides for identification and frame-to-frame tracking of each particle. This enables determination of the velocity of each particle's Brownian motion, which can then be used to calculate its hydrodynamic diameter by applying the Stokes-Einstein equation when the temperature, sampling time and sample viscosity is known[164,172]. Also the particle concentration is estimated by the software, and data are presented as a histogram of particle diameter versus particle concentration. NTA enables detection of particles with diameters down to 10-35 nm[173-175]. Thus, NTA allows for the measurement of particles considerably smaller than those detected by FC. However, NTA does not provide identification of EV phenotyping or distinction between EVs and other particle types. Although NTA includes the possibility of detection of a fluorescence signal from labeled EVs[164], this expansion of the technique is hampered by rapid bleaching of common chromophores[141] and fluorescence NTA is still regarded as being in an early stage of development[171].

Tunable resistive pulse sensing

TRPS is based on the Coulter principle, which was first documented by the Coulter brothers in 1948[176] and applied in the earliest flow cytometers for blood cell counting

in the 1950s. The Coulter principle relies on the fact that a particle that is present in an electric field in a conductive liquid will change the current flow in the electric field. When a part of the current path is constricted, the passage of a particle through the constricted area can cause a change in current flow that can be detected and measured[177]. In TRPS, an upper and a lower fluid cell are separated by a non-conductive membrane with a small aperture, a so-called 'nanopore' in it. When the upper and lower fluid cell as well as the aperture that interlink them contain a conductive liquid and a voltage is applied to the system, a continuous current of ions is established and measured as the 'baseline current'. The sample that contains the particles of interest constitutes the conductive liquid in the upper flow cell. When a particle passes through the aperture, it causes a brief increase in the impedance of the pore, reflected by a transient decrease in current, which is detected by the system and represented by a peak-shaped negative deviance from the graphically monitored baseline current. The magnitude and rate of these negative peaks are used to estimate the sizes and the concentration of the particles[166,178]. Thus, as in NTA, a histogram of particle diameter versus particle concentration is obtained, while phenotyping is not. A combination of different forces, including electrophoretic and convective flow, cause the movement of particles through the nanopore[178], and a variable pressure module can be used to add extra force[166]. Different nanopores with different aperture diameters exist and can be selected for particular samples according to the expected size range of the particles in the sample[179], and furthermore the user can adjust the diameter of the aperture by changing the stretch of the pore holder[178].

Extracellular vesicle Array

The EV Array is a recently developed method based on the protein microarray technology, which is used for identification of antigens or antibodies in biological samples. The EV Array, which has been under development since 2011[180] and was first documented in a publication from 2013[168], is prepared by printing a chosen panel of capturing agents, typically antibodies, on epoxy-coated microarray slides. The method has been tested with a range of capturing agents enabling capture of EVs by binding their surface-associated antigens. Tested capturing agents include antibodies against the tetraspanins CD9, CD63, and CD81 that are typically present on the surface of exosomes[168,181] and annexin V[181] which binds PS[171]. After drying, the printed slides are incubated with the sample containing the EVs prior to addition of biotinylated detection antibodies. Then fluorescently labeled streptavidin is applied and binds to biotin on the detection antibodies. The fluorescence intensity from the bound streptavidin, which is determined by a fluorescence scanner, is used as a semiquantitative measure of the sample's content of EV surface molecules that bind the capturing and detecting agent used in the EV Array, whereas direct counting and size determination of particles are not obtained with this method. As detection agents a combination of biotinylated antibodies against CD9, CD63, and CD81 have been tested, but also other biotinylated antibodies can be used[181]. As an open platform, EV Array can be modulated for detection of subpopulations such as TF-bearing EVs[182] or subpopulations carrying antigens that reveal their cellular origin.

CD146, CD31, and CD62E have been established as markers of endothelial cell origin in EV studies[183], while CD206 and CD163 are expressed by macrophages and dendritic cells[184]. CD14 exists on monocytes and macrophages and in low levels on neutrophils[185], while CD42a, CD42b, and CD41 are present in platelet membranes[186].

PREANALYTICAL CONSIDERATIONS

Blood sampling and plasma preparation

Regarding blood collection, the use of a tourniquet, venipuncture, and blood flow through a needle with a small diameter into a vacuum blood collection tube may cause hemolysis, endothelial damage, or platelet activation, which may lead to an increase in the EV number and other changes in the population of EVs detected in the sample[146]. If *serum* is used for EV analysis, over 50 % of the detected EVs may be accounted for by platelet-derived EVs released after the blood collection during clot formation[123]. In an ISEV position paper[123] it was stated that *plasma* is the physiological medium of EVs in the blood and that most studies on circulating EVs had been performed on plasma[123] which has been recommended for most applications[171]. The type of anticoagulant used for plasma sampling, the time interval between blood collection and centrifugation, and the temperature and agitation that the blood is exposed to can affect the results of subsequent EV analysis[146,171]. Meticulous removal of platelets is important since remaining platelets release EVs during freezing and thawing of plasma[171] but, on the other hand, centrifugation required to remove all platelets is likely to also remove large EVs[123]. EVs in plasma seem to be stable during one freeze-thaw cycle, but it has been recommended to avoid repeated freeze-thaw cycles[171]. General recommendations on practical preanalytical handling of blood samples for EV analysis were given in a publication released by Lacroix et al. at about the time of the beginning of this PhD project[187] and in a recent review on EV analysis[171].

EV isolation

Apart from preparation of platelet free plasma (PFP) by removal of blood cells and platelets, which has been recommended to be done by applying two subsequent centrifugation steps of 2500 g for 15 minutes[171,187], it would for some purposes be desirable to isolate EVs entirely. However, all isolation methods affect the concentration of EVs and no method is capable of isolating EVs only[171]. Typically co-isolated components include protein aggregates and some types of lipoproteins[171]. A common method of EV isolation is differential centrifugation. This approach allows for pelleting EVs and for some degree of separation of large and small EVs but also holds considerable limitations since it does not provide complete isolation of EVs from protein aggregates and lipoprotein particles and furthermore tends to damage EVs during the final ultracentifugation step and result in aggregation of EVs[188,189] and poor EV recovery[171]. Density gradient centrifugation enables removal of protein contaminants but is time-consuming[123,171] and does not provide complete isolation of EVs from small lipoproteins[190,191]. More recently, a much less time-consuming method termed size exclusion chromatography (SEC) was proposed for EV

purification[192]. While SEC does not tend to induce aggregation or biophysiological changes of EVs, it dilutes them and implies coisolation of EVs, protein aggregates, and large lipoproteins[171]. Other isolation methods that may be applied include filtering although it may damage EVs[123], precipitation although it is unable to isolate pure EVs, and immunocapture which may be used to capture subpopulations of EVs[171].

Regarding the unfavourable side effects of attempts of EV isolation, Mooberry et al. recommended that FC-based EV analysis should be performed on plasma[146].

ANALYTICAL CONSIDERATIONS

While the above described techniques for EV analysis can provide indications of the amounts, sizes, and phenotypical characteristics of EVs in a solution, the data obtained by their use needs to be interpreted with reservations.

The heterogeneity of EVs present in a biological sample can be illustrated by the proposition that the smallest and the largest of them typically differ 25-fold in size, 300,000-fold in concentration, 20,000-fold in volume, and 10,000,000-fold in light scattering properties, which complicates exact EV enumeration[193]. Light scatter-based methods for EV measurement, such as FC and NTA, are also challenged by the lack of a reference material with a refractive index equalling that of EVs[194,195]. In FC, EV measurements are typically based on scatter signals from polystyrene or silica beads although these have a different refractive index than EVs, which inevitably compromises both EV size and concentration measurements[196]. In NTA, the measured concentration depends on the brightness of the scattering particles, which means that the refractive index, and furthermore the size distribution of the particles present in the sample, will affect concentration measurements[196]. A particle that scatters less light than the limit of detection, will not be measured[197]. For standardization of NTA-based as well as FC-based EV measurements a biological standard would be desirable[195], and attempts to identify candidates for such new reference materials are going on[196].

When EVs are analyzed by FC, the possibility of coincidence events, also termed ‘swarm detection’, exists, meaning that multiple EVs simultaneously passing the laser beam are counted as one event, which affects the measured event rate[169,198]. In this way a single small EV, which does not by itself scatter enough light or emit enough photons to be detected by light scatter or fluorescence measurement, respectively, may together with other EVs generate a signal that exceeds the detection threshold[141]. Flow cytometers differ in optical configuration and sensitivity, and as is the case with other methods for EV measurement, standardization of FC-based EV measurement has not been obtained, which makes data comparison between laboratories problematic[141].

While FC because of its limitations regarding measurement of the smaller EVs, only casts light on the tip of the iceberg of the EV population, NTA and TRPS allow for some exploration of the ‘bulk’ of the iceberg. However, the concentration measurements obtained by these methods still strongly depend on the lower size limit of detection[141]. Since no reference material with the ideal refractive index

exists, NTA cannot be accurately calibrated for EV measurment, which implies some inherent error in the obtained results. Since the nanopores used in TRPS display considerable pore-to-pore variation and the baseline current through a pore varies over time, the minimum detected size with TRPS is inconstant[178].

EV Array does not provide size measurements of EVs and as opposed to the other three methods described here, it does not detect single EVs. Instead it semiquantifies the EVs by means of the overall fluorescent intensities from the detection agents bound to the EV-associated proteins present without taking into account how many copies of each antibody-bound protein that are present on each particle. Also in FC, the amount of the antigen of interest present on a single particle is an analytical issue, since only vesicles that carry a sufficient amount to generate a detectable fluorescence signal are detected[197], and the relationship between the detected fluorescence signal and the number of epitopes is complex[141].

While EV Array as well as FC can provide some phenotypical information on the EVs in a sample based on fluorescent labeling of proteins, these approaches imply the introduction of potential sources of error and variance related to factors such as antibody specificity, antibody aggregate formation, low signal-to-background ratio, and variability in staining intensity[194]. Also, it should be noted that no generic label for all types of EVs exists[141] and the lack of such an invariant 'household marker' complicates label-based EV quantification[168]. PS has been used as an MV marker in several studies on plasmatic EVs, but a significant number of MVs do not appear to be PS-positive[119,199], and experimental conditions, such as calcium concentrations affect the binding to PS when annexin V is used as a probe[200]. Lactadherin has been demonstrated to be more sensitive than annexin V for PS detection[201], and since lactadherin binds PS in a calcium independent manner, it has been stated that lactadherin can be used to detect PS-exposing MVs directly in citrate-anticoagulated plasma samples[124].

Lipoproteins

Lipoprotein subtypes and functions

Lipoproteins are particles involved in the transport of lipids between tissues. As opposed to EVs, lipoproteins only have a single layer of phospholipids in their membrane[191], which also contains free cholesterol and different types of apolipoproteins, and the surface of a lipoprotein particle resembles the external half of a cell membrane[202,203]. The hydrophilic surface allows for the transport in plasma of hydrophobic molecules, namely triglycerides (TGs) and cholesterol esters, contained in the hydrophobic core of lipoproteins[203].

Subclasses of lipoproteins termed chylomicrons, chylomicron remnants, very low density lipoproteins (VLDLs), intermediate density lipoproteins (IDLs), low density lipoproteins, (LDLs), and high density lipoproteins (HDLs) play their different parts in the redistribution of dietary lipids in the body via the exogenous and endogenous lipoprotein pathway and the reverse cholesterol transport pathway[202].

Chylomicrons are large triglyceride rich lipoproteins (TRLs), each containing one apolipoprotein B-48 (ApoB-48) molecule. They are formed in the intestinal cells upon absorption of monoacylglycerols, fatty acids, and cholesterol from the intestinal

lumen after food ingestion. The diameter of chylomicrons ranges from 75 to 1200 nm[202,204] and they are especially large and TG-rich when a fat-rich meal has been consumed[202]. Upon formation, chylomicrons are secreted into the lymph and via ductus thoracicus they reach the circulation where they are metabolized by lipoprotein lipase (LPL) present in muscle and adipose tissue. LPL hydrolyzes TG in chylomicrons, and the resulting *chylomicron remnants* have a diameter between 30 and 80 nm and contain less TG, while they expose apolipoprotein E, which allows for receptor-mediated uptake of chylomicron remnants in hepatocytes[202]. This so-called exogenous lipoprotein pathway, starting with the formation of chylomicrons in the intestine and ending with their uptake by the liver, normally enables metabolism of considerable amounts of dietary fat and transfer of dietary fatty acids to muscle cells and adipocytes with only modest resulting increases in plasma TG levels[202].

VLDLs are TRLs, most of which have a diameter between 27 and 60 nm, although some of them may have a diameter of up to 200 nm[205,206]. They are formed in the liver in a process that involves transfer of TG and cholesterol esters to apolipoprotein B-100 (ApoB-100). VLDLs are secreted from hepatocytes into the blood and reach the peripheral tissues where their TG is hydrolyzed by LPL, resulting in their transformation into *IDLs* with a smaller diameter of 23-35 nm[202,205,206]. IDLs contain less TG than VLDLs and are thus relatively enriched in cholesterol esters. IDLs are subjected to further TG depletion via hydrolysis and some of their apolipoproteins are transferred to other lipoprotein particles resulting in the transformation of the IDLs to *LDL*s, which mainly consist of cholesterol[202] and have diameters between 18 and 23 nm[205,206]. LDLs are taken up from the circulation by LDL receptors in the liver and several other tissues. Throughout this endogenous lipoprotein pathway that involves metabolism of VLDLs to IDLs and then LDLs, each of these three subtypes of lipoproteins contains exactly one ApoB-100 molecule per lipoprotein particle[202].

In the postprandial phase, the concentration of ApoB-48-bearing particles, which include chylomicrons and chylomicron remnants, increase significantly, and the concentrations of ApoB-100-bearing particles increase even more[207,208].

The formation of *HDL*s relies on the synthesis of Apolipoprotein A-I (ApoA-I) which is mainly produced in the hepatocytes and enterocytes and then secreted to the circulation where it initially acquires cholesterol and phospholipids being effluxed from hepatocytes and enterocytes. The thus formed HDLs then also acquire cholesterol and phospholipids from cells such as muscle cells and adipocytes, and furthermore from chylomicrons and VLDLs from which the HDLs also take up some apoliproteins but not ApoB-100[202]. The cholesterol contained in HDLs, which have diameters between 5 and 12 nm[202,203], is mainly delivered to the liver. This so-called reverse cholesterol transport is necessary for most cell types in order to decrease their cholesterol content and is probably important for the prevention of atherosclerosis[202].

Interference of lipoproteins on extracellular vesicle analysis

Since EVs and lipoproteins have overlapping size ranges, and lipoproteins are present in plasma in numbers that are probably significantly higher than EV numbers[171], it seems plausible that lipoproteins may interfere with EV analysis methods that rely

on concentration measurement of particles within a given size range, such as NTA and TRPS. Dragovic et al. performed studies which indicated that most particles detected by light scatter-based NTA of platelet-poor plasma samples are lipoproteins[164], and Gardiner et al. suggested that lipoproteins account for more than 98 % of the particles detected by NTA in PFP that has not been ultracentrifuged[195].

However, even when FC with phenotypical characterization of the detected particles is used for EV measurement, the results may be influenced by lipoproteins present in the sample. In 2016, Sódar et al. published data showing that most of the particles detected by FC in PFP exposed lipoprotein markers in the form of ApoB-100/-48 and that such ApoB-positive events highly outnumbered EV marker-exposing events in the form of CD9-positive, CD41a-positive, and annexin V-binding events. When results on PFP obtained from blood collected in the fasting state were compared with results on PFP obtained from blood collected in the postprandial state, it was evident that in the postprandial PFP samples the concentrations of ApoB-exposing particles were significantly increased, while CD9- and CD41-positive events decreased significantly, and PS-positive events decreased unsignificantly[191]. In the same study, when FC was applied on isolated MV preparations obtained from PFP by differential ultracentrifugation and gravity driven size filtration, the detected number of annexin V-binding events significantly decreased in the postprandial as compared to the fasting state. While it had previously been reported that HDL particles co-purify with exosomes[190], the study by Sódar et al. unexpectedly revealed that ApoB-100-bearing particles co-purified with MVs and with exosomes isolated by the abovementioned purification techniques. Moreover, when commercially acquired purified LDL was mixed in vitro with cell-line derived MVs and exosomes, LDL particles were shown to bind extensively to both types of EVs, and it was suggested that binding of lipoproteins to EV surfaces may to some degree hinder the detection of EV markers in the postprandial state[191].

"How could we miss lipoprotein particles until now? Why did we not detect them before?"

(Sódar et al., 2016)[191].

The quote from Sódar et al. stresses the authors' amazement of their finding that ApoB-bearing lipoproteins co-purify with EVs and may profoundly affect EV measurements even when particle phenotyping by FC is applied.

Surely, the potential effect of blood contents of lipoproteins, and thus prandial state at the time of blood sampling, on EV measurements has been a matter of concern in this PhD project, reflected in the work presented in paper 1, 2, and 3.

AIMS AND HYPOTHESES

The primary aim of this PhD project was to gain insight into the mechanisms of thrombus formation in AF patients. The nomination of procoagulant EVs as a potential contributing factor to AF-related thrombogenicity gave rise to a number of secondary aims related to the discipline of EV analysis.
While study 4 was the one that was carried out last, it was the hypotheses of study 4 that set the scene for the entire body of scientific work performed within the framework of the PhD project, and therefore these will be presented first.

STUDY 4

Based on the background information that

- AF is a major risk factor of thromboembolism[32]
- The LAA is the most common site of intra-atrial thrombus formation in AF patients[83]
- AF results in changed blood flow, including reduced flow velocity, in the LAA[85]
- The LAA endothelium can be damaged and infiltrated by inflammatory cells in AF patients[105]
- TF and vWF can be overexpressed in the LAA endothelium in AF patients[105]
- Cell stress and activation can induce cellular release of EVs[124]
- EVs can have procoagulant capacity, especially subtypes that expose TF[154]

we reasoned that conditions which may promote procoagulant EV release and accumulation in the LAA lumen appear to be present in AF patients and established the hypothesis that:

Elevated levels of procoagulant EVs exist in blood located in the LAA as compared to venous blood in patients with AF.

However, while some accumulation of procoagulant EVs potentially derived from cells in the LAA wall may take place due to blood stasis, the actual extent of blood exchange between the LAA lumen and the rest of the circulation is not well-known and may be substantial. Furthermore, it is conceivable that AF may induce release of EVs to the blood from cells located outside the LAA, such as the main part of the LA. Based on these considerations and the previous reports on increased levels of TF[102], vWF[87], and other hemostatic markers[87] in *venous* blood in patients with AF, we also hypothesized that:

Elevated blood levels of procoagulant EVs exist in AF patients as compared to subjects without AF.

Thus, the aim of this study was to compare the amounts of EVs, including EVs exposing TF and markers of platelet, leukocyte, or endothelial cell origin, in venous blood and blood drawn directly from the LAA in patients with and without AF. No patients with valvular AF were included, because it has been indicated that mitral valve disease in itself induces endocardial damage and potentially thrombogenic changes in the LAA[107]. Apart from EVs, we aimed to evaluate levels of circulating TF, vWF, PPLs, NETs, and total submicron particles in the collected blood samples.

OVERALL AIM OF STUDY 1, 2, AND 3

As it appears from the background section, the field of EV analysis holds several challenges and lacks standardization[42,141]. Thus, in order to pursue the hypotheses of study 4, it was necessary to establish, gain experience with, and evaluate strategies for EV measurement, which constituted the overall aim of study 1, 2, and 3.

Considering the background information that

- plasma is the physiological medium of EVs in blood[123]
- remaining platelets in plasma release EVs during freezing and thawing of plasma[171]
- all isolation methods affect the concentration of EVs and no method is capable of isolating EVs only[171]

we found it relevant to focus on strategies for EV measurement on PFP instead of isolated EVs or customary plasma.

The specific aims of study 1, 2, and 3 are formulated in the following.

STUDY 1

As NTA[163] and TRPS[179] have been nominated as valuable methods for measurement of smaller EVs in biological samples, supplementing methods such as conventional FC that may only enable detection of the 'tip of the iceberg' of EV populations[170], we planned to establish NTA and TRPS in our laboratory. However, since lipoproteins in the same size range as small EVs[195,206] exist in plasma at any given time and are especially abundant in the postprandial phase[207,208], lipoprotein particles plausibly represent a substantial source of interference with EV measurements on PFP by NTA and TRPS. By preparing PFP by two centrifugation steps as recommended by Lacroix et al.[187] we should avoid EV release from platelets during the freeze-thaw cycle. However, we found it relevant to examine whether or not freezing and thawing PFP prior to measurement would affect the particle population.

Thus, the aims of study 1 were to

- evaluate the analytical linearity and analytical variation of submicron particle measurements by NTA and TRPS

- estimate the inter- and intra-individual biological variation of submicron particles in PFP as measured by NTA and TRPS
- evaluate the impact of a freeze-thaw cycle on the submicron particle population in PFP as measured by NTA and TRPS
- evaluate the impact of prandial state on the submicron particle population in PFP samples as measured by NTA and TRPS

As such, study 1 was of descriptive nature, but the two last-mentioned aims were prompted by the underlying hypotheses that:

A freeze-thaw cycle significantly affects the submicron particle population as measured by NTA and TRPS.

and

TG-rich lipoproteins appearing in the postprandial state significantly increase submicron particle concentrations as measured by NTA and TRPS.

Furthermore, as part of study 1, we performed an additional investigation on SEC fractions of PFP that were relatively enriched in EV content compared to HDL and protein content as described by Boing et al.[192] In this additional study we aimed to examine if particle levels in these particular SEC fractions were affected in the same way by food ingestion and a freeze-thaw cycle as the samples in the main study.

STUDY 2

As discussed in paper 1[209], ApoB-bearing lipoproteins plausibly account for a non-negligible share of the particles detected by NTA in PFP from fasting blood samples, even in EV-enriched fractions obtained by SEC. Hence, we designed and conducted study 2 as a proof of concept study[210] in which we aimed to explore the potential of removing interfering lipoproteins from PFP by use of anti-ApoB antibody-coated magnetic beads prior to EV analysis by NTA.

The corresponding hypothesis was:

Lipoproteins interfering with EV analysis by NTA can be removed from PFP by use of magnetic beads coated with anti-ApoB antibodies.

STUDY 3

Bearing in mind that

- the effect of food intake on the EV population in the blood is not well known[123], although elevated levels of EVs derived from endothelial cells[134] and platelets[135] in the postprandial state have been reported

- postprandial coagulation activation has been reported[110]
- elevated postprandial levels of circulating TF have been described[111]
- lipoproteins may interfere with FC-based EV measurement[191]

we conducted study 3 in which we aimed to

- evaluate the impact of prandial state on PFP concentrations of PS-exposing submicron particles as measured by FC, including subtypes staining positive for TF or markers of platelet or endothelial cell origin
- evaluate the impact of in vitro addition of lipoproteins to fasting PFP on PFP concentrations of PS-exposing submicron particles measured by FC, including subtypes staining positive for TF or markers of platelet or endothelial cell origin
- evaluate the impact of prandial state on PFP content of EVs as measured by EV Array, including subtypes exposing PS, TF, or markers of platelet or endothelial cell origin

The corresponding hypotheses were:

Prandial state affects PFP concentrations of PS-exposing submicron particles measured by FC, including subtypes staining positive for TF or markers of platelet or endothelial cell origin,

In vitro addition of lipoproteins to fasting PFP mimics the effect of food ingestion on PFP concentrations of PS-exposing submicron particles measured by FC, including subtypes staining positive for TF or markers of platelet or endothelial cell origin,

and

Prandial state affects PFP content of EVs as measured by EV Array, including subtypes exposing TF, PS, or markers of platelet or endothelial cell origin.

Moreover, we aimed to evaluate TF antigen and TF activity levels in PFP from fasting and postprandial blood samples.

MATERIALS AND METHODS

STUDY POPULATIONS

STUDY 1 AND 3

The same study population was used for study 1 and study 3. 20 adult subjects (7 males and 13 females) aged 26-64 years were included after being considered healthy, based on a medical questionnaire and blood tests that included hematology tests, liver and kidney function tests, and blood glucose levels.

STUDY 2

10 healthy individuals (4 females and 6 males) aged 27-60 years were included.

STUDY 4

7 patients with paroxysmal, nonvalvular AF (aged 45-81 years), 6 patients with persistent or permanent nonvalvular AF (aged 67-76 years), and 12 controls patients (aged 48-80 years) without AF were included. The control group and the AF group were both comprised of patients who had been referred to either coronary artery bypass grafting or aortic valve surgery at the Department of Cardiothoracic Surgery, Aalborg University Hospital.

BLOOD SAMPLING AND SAMPLE HANDLING

STUDY 1 AND 3

Blood for study 1 and 3 was sampled from the median cubital vein through a Vacuette Safety Blood Collection Set (Greiner Bio-One, Kremsmünster, Austria) with a 21 gauge needle and a 19 cm flexible tube after application of a light tourniquet. Fasting samples were collected at 8.15 am. At 8.30 am the study participants were served a non-standardized breakfast and postprandial samples were collected at 9.45 am. Blood destined for PFP preparation was collected in 9 mL 3.2 % sodium citrate plastic tubes (Greiner Bio-One) after the first 3.5 mL of blood obtained from the intravenous access had been discarded. Blood for routine tests, such as lipid measurements, was collected into standard tubes for the respective tests at Aalborg University Hospital and analyzed on the day of blood sampling.
PFP was prepared by applying two centrifugations at 2500 g for 15 minutes at room temperature. The first centrifugation was initiated within 30 minutes after the blood sampling. When collecting supernating plasma for transferal to a clean plastic tube after the first centrifugation, 10 mm of plasma was left uncollected above the buffy coat. Fresh PFP from all patients was analyzed by NTA and TRPS on the day of blood sampling. The remaining PFP was divided into aliquots for the different analyses and frozen and kept at -80 °C until the day of analysis.

STUDY 2

In study 2, fasting blood samples were collected and PFP was prepared in the same way as in study 1 and 3. PFP was kept at -80 °C until the day of experimental lipoprotein removal and analysis. For removal of ApoB-exposing lipoproteins from PFP, we established the 'bead procedure' in which PFP was incubated with anti-ApoB antibody-coated magnetic beads (prepared as described under 'Additional materials') and mixed under rotation for 120 minutes, allowing for antibody-based binding of the magnetic beads to VLDL, LDL, and IDL particles, and chylomicrons. Following this incubation, PFP was separated from the beads by use of a DynaMag-2 (Life Technologies, Carlsbad, CA, USA) magnet before analysis.
Blood for measurement of lipid levels was collected into standard tubes for these tests at Aalborg University Hospital and analyzed on the day of blood sampling.

STUDY 4

In study 4, 3.5 mL Vacuette 3.2 % sodium citrate plastic tubes (Greiner Bio-One, Kremsmünster, Austria) were used for pre- and intraoperative collection of blood for PFP preparation. All study participants were preoperatively fasting at the time of blood sampling. Immediately before commencement of surgery, 3 tubes of venous blood for PFP preparation were sampled from a central vein via a central venous catheter. After sternotomy, but immediately before systemic heparinisation, another 3 tubes of central venous blood were sampled intraoperatively along with 3 tubes of blood sampled through a cannula which was inserted in the LAA. A 75 cm Luer Lock Extension tube (Praxisdienst, Longuich, Germany) interlinked the cannula and the blood sampling tubes. PFP was prepared in the same way as in study 1, 2, and 3, except that the time period between blood sampling and initiation of first centrifugation was up to 60 minutes. NTA analysis was performed on the day of blood sampling, while PFP for the remaining analyses was kept at -80 °C until the day of analysis. Blood for measurement of plasma lipid levels was collected before surgery into standard tubes for these tests at Aalborg University Hospital and analyzed on the day of blood sampling.

BLOOD ANALYSIS

NANOPARTICLE TRACKING ANALYSIS

NTA was performed in study 1, 2, and 4. In all three studies the same NTA instrument, settings, and work routines were used. The applied NTA device was a
LM10-HS (Malvern Instruments Ltd, Malvern, UK) with a Luca DL-658M-OEM EMCCD camera (Andor Technology, Belfast, UK) and a 405 nm laser. NanoSight NTA 2.3 was used for data processing. As PFP samples were diluted several hundred times in Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza, Basel, Switzerland) when analyzed by NTA, sample viscosity was set to the corresponding viscosity for water at the given room temperature. Samples were loaded manually with a syringe into the sample chamber. The microscope field of view was positioned in the area where

the particles were most clearly visualized and video recordings with a length of 60 seconds were made. Background signal was subtracted before processing. The camera shutter was set to 500, camera gain to 300, and detection threshold to 3. With regard to minimum track length, blur, and minimum expected particle size automatic settings were selected.

TUNABLE RESISTIVE PULSE SENSING

TRPS was applied in study 1 and was performed using a qNano (Izon, Oxford, UK). An NP200 nanopore (Izon) was mounted and kept at a constant 47 mm stretch during analysis. A constant pressure of 7 cm H_2O was applied on the system. A qNano CPC200B microsphere concentrate (Izon) which had been diluted 1000-fold in filtered DPBS was used for calibration. Measurements were continued for at least 180 seconds or until at least 500 particles had been detected. Baseline current during analysis was between 70 and 150 nanoampere.

APOLIPOPROTEIN B ASSAY

In study 2, ApoB was quantified before and after the experimental removal of ApoB-exposing lipoprotein particles by applying Apolipoprotein B (APOB) Human ELISA kit (Abcam, Cambridge, UK) and measuring the absorption at a wavelength of 450 nm using a SpectraMax M2 Microplate Reader (Sunnyvale, CA, USA).

EXTRACELLULAR VESICLE ARRAY

EV semiquantification by EV Array was performed in study 2, 3, and 4, based on the method originally described by Jørgensen et al.[168]

In study 2, the applied EV Array featured a mixture of antibodies against CD9, CD63, and CD81, i.e. the so-called 'exosome cocktail', both as capturing and detecting agents and was used to estimate the extent of a potential loss of small EVs as a side effect of lipoprotein removal.

In study 3, capturing agents included lactadherin and antibodies against CD9, CD63, CD81, CD41, CD146, CD62E, and TF, while the exosome cocktail was used as detecting agent. With this design, we aimed to estimate the amounts of EVs exposing CD9, CD63, and CD81 in general and subtypes that also exposed PS, TF, or markers of platelet or endothelial cell origin, in the fasting and postprandial state.

In study 4, a broader panel of capturing agents was applied. These included antibodies against CD9, CD63, CD81, and annexin V along with a range of antibodies against cell type-specific CD molecules, including CD42a, CD42b, CD41, CD31, CD146, CD62E, CD206, CD163, and CD14, and moreover two clones of anti-TF antibodies (the TF9-10H10 clone and the HTF-1 clone, respectively). A combination of antibodies against CD9 and CD81 was used as detecting agents. With this version of EV Array we aimed to compare levels of tetraspanin-bearing EVs, and subtypes exposing PS, TF, and markers of platelet, endothelial cell, or leukocyte origin. Parallelly, we applied an additional version of EV Array with the abovementioned range of antibodies against cell type-specific CD molecules as catching agents, combined with anti-TF antibodies as detecting agent in order to study which cell types may

contribute to PFP content of TF-bearing EVs. Both of these versions of the EV Array were applied on PFP from pre- and intraoperative venous blood samples as well as PFP from blood samples drawn directly from the LAA in both the AF patient group and the control group.

FLOW CYTOMETRY

FC was applied in study 3 using a BD FACSAria III High Speed Cell Sorter (BD Biosciences, San Jose, CA) and applying an approach that was based on a method previously described by Nielsen et al.[167] TruCount™ tubes (BD Biosciences) with a lyophilised pellet releasing a known number of fluorescent beads serving as an internal standard enabling determination of particle concentration were used. Fluorescein isothiocyanate-conjugated lactadherin was applied as a marker of PS-positive events. To identify PS-positive events that also exposed platelet markers, particles were further labeled with 3 µL of allophycocyanin-conjugated anti-CD41 antibodies. Phycoerythrin (PE)-conjugated anti-CD146 antibodies, and PE-conjugated anti-CD62E antibodies, respectively, were added to label PS-positive particles which also carried markers of endothelial cell origin. TF-exposing events were identified by labeling with PE-conjugated anti-TF antibodies. Isotype controls that matched the respective antibodies against the abovementioned proteins were applied as negative controls. By use of a blend of size-calibrated fluorescent polystyrene beads with sizes ranging from 0.2 to 0.9 µm, a size gate in a FSC and SSC setting allowing for detection of particles with a diameter of approximately 0.2–1.0 µm, was established. FC was performed with a maximal analysis rate of 20,000 events per second and each run was ended when event counts reached 500,000.

TISSUE FACTOR ACTIVITY ASSAY

For TF activity measurements, which were performed in study 3 only, Tissue Factor Human Chromogenic Activity Assay Kit (Abcam) was applied, and the absorbance at a wavelength of 405 nm was measured using the SpectraMax M2 Microplate Reader.

TISSUE FACTOR ANTIGEN ASSAY

TF antigen concentrations were measured in study 3 and 4 with Imubind anti-human Tissue Factor ELISA (Sekisui Diagnostics, Lexington, MA, USA) applying the SpectraMax M2 Microplate Reader for absorbance measurement at a wavelength of 450 nm.

In study 3, an additional method for TF antigen measurement, represented by a modified version of EV Array with anti-TF antibodies as both capturing and detecting agents, was applied.

PROCOAGULANT PHOSPHOLIPID DEPENDENT CLOTTING TIME

To evaluate the content of PPL in PFP in study 3 and study 4, STA Procoag PPL (Diagnostica Stago, Asnières, France) was run on a STA-Compact Coagulation Analyzer (Diagnostica Stago). In this assay, the clotting process in a mixture of the

PFP sample of interest and human plasma from which the procoagulant phospholipids have been removed is iniatied by addition of activated bovine FX. The PPL-dependent clotting time (PPL-CT) is measured and reported as the test result.

VON WILLEBRAND FACTOR ASSAY

In study 4, the concentrations of vWF antigen in PFP were measured with vWF Ag (Siemens Healthineers, Erlangen, Germany) on a Sysmex CS2100i (Siemens Healthineers).

CIRCULATING CELL FREE DEOXYRIBONUCLEIC ACID ASSAY

In study 4, PFP concentrations of cell free DNA (cf-DNA) were determined as a surrogate measure of NETs. DNA was labeled with SYTOX Green nucleic acid stain (Thermo Scientific, MA, USA) in a Nunc 96-Well Polypropylene MicroWell Plate (Thermo Fisher). Light at a wavelength of 485 nm was applied for excitation. Emission at a wavelength of 520 nm was detected with a FLUOstar OPTIMA microplate reader (BMG LABTECH, Ortenberg, Germany).

ROUTINE BLOOD TESTS

Lipid levels in study 1, 2, 3, and 4 were measured on a Cobas 8000 Modular Analyzer (Roche Applied Science, Penzberg, Germany). Supplementary glucose measurements and routine liver and kidney function tests for study 1 and 3 were also measured on the Cobas 8000, while hematology tests were performed on an Advia 2120 Hematology System (Bayer Healthcare, Leverkusen, Germany).

ADDITIONAL MATERIALS

COLUMNS FOR SIZE EXCLUSION CHROMATOGRAPHY

In study 1, SEC was performed using 10 mL qEV columns (Izon). After washing the qEV column with 10 mL of DPBS, 0.5 mL of PFP was loaded followed by elution with DPBS, and 0.5 mL fractions were collected. Fraction 9 and 10 were expected to be relatively EV-enriched, i.e. the HDL and protein content was expected to be markedly more reduced than EV concentration, as reported by Böing et al[192].

POLYSTYRENE BEAD SOLUTIONS FOR TRPS VARIATION STUDY

In study 1, as a supplement to the TRPS between day variation study on PFP, an additional between day variation study on a microbead solution consisting of qNano SKP200B polystyrene microsphere concentrate (Izon) diluted in filtered DPBS was performed.

LOW DENSITY AND VERY LOW DENSITY LIPOPROTEIN ISOLATES

In study 2, Human Low Density Lipoprotein (LDL) 5 mg/mL and Human Very Low Density Lipoprotein (VLDL) 1 mg/mL (Kalen Biomedical, Montgomery Village, MD,

USA), diluted in filtered DPBS to a concentration within the linear range of NTA concentration measurement, were used for testing the efficacy of the magnetic bead procedure for lipoprotein removal.
In study 3, the same lipoprotein isolates from Kalen Biomedical were used for in vitro addition of lipoproteins to PFP obtained in the fasting state. The LDL isolate was added in amounts that had been demonstrated to increase TG concentration by 0.1 mmol/L and cholesterol concentration by 2.2 mmol/L. The VLDL isolate was added in amounts that had been demonstrated to increase TG concentration by 0.2 mmol/L and cholesterol concentration by 0.2 mmol/L.

ANTI-APOLIPOPROTEIN B ANTIBODY-COATED MAGNETIC BEADS

Prior to the lipoprotein removal step in study 2, the anti-ApoB antibody-coated magnetic beads used in study 2 were prepared in our laboratory. In brief, Dynabeads Protein G 30 mg/mL (Life Technologies, Carlsbad, CA, USA) were transferred from the stock solution to a microtube (Sarstedt, Nümbrecht, Germany). By use of the DynaMag-2 magnet, the beads were separated from the solution, and the supernatant was removed. Polyclonal anti-human Apoliprotein B-48/-100 antibodies 1 mg/mL (Meridian, Life Science, Inc., Memphis, TN, USA) were diluted in DPBS with 0.02 % Tween-20 (Bio-Rad, Hercules, CA, USA) and incubated with the magnetic beads under rotation for 10 minutes. During this incubation period, fragment crystallizable (Fc) regions of the antibodies bind to Protein G on the beads. The hereby prepared antibody-coated beads were then separated from the solution by use of the magnet, and the solution was discarded. Next, the antibody-coated beads were washed three times, using DPBS with 0.02 % Tween-20, before a final application of the magnet and removal of the DPBS used for the third wash.

STATISTICS

In all four studies, the Wilcoxon matched pairs signed rank test was used for paired data analysis such as analyte levels in the fasting versus the postprandial state, particle concentrations before versus after the bead procedure, and analyte levels in intraoperative samples drawn from the LAA versus venous samples.
For correlation analysis Spearman's correlation was applied in all four studies.
In study 3, we also applied parametric comparison and correlation methods, namely paired t-test and Pearson's r, based on the Anderson-Darling test for normality.
For comparison of independent groups, i.e. the AF patient group versus the control group in study 4, the Mann-Whitney U test was used.
Two-sided p-values were given, and p-values below 0.05 considered statistically significant.
In study 1, The 95 % reference intervals were estimated using parametric analysis of log-transformed and untransformed data for particle concentration and particle size data, respectively. For evaluation of analytical linearity, linear regression was performed on measured concentrations plotted against expected concentrations. Expected concentrations were calculated as the mean of all raw concentrations of

measurements within the ranges considered linear, based on visual assessment, divided by the dilution factor.
In study 1 and 3, pooled CVs were calculated as the square root of the mean of the squared CVs.
Software applied for statistical analysis was R version 3.1.1 for Windows (R Foundation for Statistical Computing, Vienna, Austria), Analyse-it version 2.24 (Analyse-it Software Ltd, Leeds, UK), and GraphPad Prism, version 6.01 (GraphPad Software, Inc., La Jolla, CA, USA).

ETHICAL CONSIDERATIONS

Study 4 occasioned ethical considerations with regard to potential risks and inconveniences for the AF patients and the control patients. After sternotomy blood was drawn from the LAA via a cannula inserted at the base of the LAA. Insertion of the cannula entailed a minimal risk of bleeding. In case of bleeding, the surgeon would suture the cannula insertion site with a single suture and the bleeding volume would be maximally a few mL. Venous blood from AF patients and control patients was drawn through a central venous catheter and thus did not necessitate venipuncture. Apart from the blood samples, LAAs routinely removed from AF patients during cardiac surgery in order to prevent thromboembolism, and a small piece of RAA tissue from both AF patients and control patients routinely removed from patients connected to the cardiopulmonary bypass machine (heart-lung machine) were collected and stored in a biobank in preparation for future studies. As these pieces of atrial appendage tissue were removed from the patients independently of study participation, it did not entail additional health risk for the patients.
The results obtained during study 4 did not affect the individual therapy of the participating patients, so the included patients did not benefit directly from participating in the study. However, the potential benefit of identifying possible novel biomarkers for risk of thromboembolism in the increasing group of AF patients was considered to outweigh the risks and inconveniences for the included patients.
The patients received written information on the study after the decision to undergo coronary artery bypass grafting or aortic valve surgery had been made. Oral information on the study was given with the room door closed by a cardiothoracic surgeon on the day the patient arrived at the Department of Cardiothoracic Surgery. The patients were offered time for deliberation regarding informed consent until the evening of the day before the procedure was performed and were informed that they had the right to withdraw the informed consent at any time.
Participants in study 1, 2, and 3 underwent an overnight fast and venipuncture of the median cubital vein. The participants were informed that venipuncture entails a minimal risk of infection, hematoma, and nerve lesion.
The studies were approved by the The North Denmark Region Committee on Health Research Ethics and The Danish Data Protection Agency.

RESULTS

STUDY 1

ANALYTICAL LINEARITY

We observed linearity with a coefficient of determination (R^2) of above 98 % within the particle concentration range of 1.0-10.0 x 10^8 for NTA and within the particle concentration range of 1.0 x 10^8 – 1.8 x 10^9 particles/mL for TRPS.

ANALYTICAL VARIATION

Nanoparticle tracking analysis
We found a between-day coefficient of variation (CV) of 6.4 % for particle concentration and 9.3 % for mean particle size in PFP samples. The within-run CV was 7.0 % for particle concentration and 6.8 % for mean particle diameter.

Tunable resistive pulse sensing
We observed a between-day CV of 24.1 % for particle concentration and 6.1 % for mean particle size in PFP, while the corresponding within-run CVs were 5.6 % and 1.4 %, and within-day CVs were 5.0 % and CVs 1.5 %, respectively.
In the additional between-day variation study on a microbead solution the CV for particle concentration was 5.9 % and the CV for mean diameter was 2.3 %.

INTER-INDIVIDUAL VARIATION

Nanoparticle tracking analysis
Estimated 95 % reference range of particle concentration was 1.4 x 10^{11} – 1.2 x 10^{12}.
Estimated 95 % reference range of mean particle diameter was 45.5–76.0 nm.

Tunable resistive pulse sensing
Estimated 95 % reference range of particle concentration was 1.8 x 10^8 – 1.6 x 10^9.
Estimated 95 % reference range of mean particle diameter was 178.5–283.2 nm.

INTRA-INDIVIDUAL VARIATION

Nanoparticle tracking analysis
Within-subject CV for particle concentrations was 33.6 %.
Within-subject CV for mean particle diameter was 16.2 %.

Tunable resistive pulse sensing
Within-subject CV for particle concentrations was 20.9 %.
Within-subject CV for mean particle diameter was 10.3 %.

FASTING VERSUS POSTPRANDIAL SAMPLES

Nanoparticle tracking analysis

In all study participants, we observed higher particle concentrations in PFP from the postprandial blood sample than from the fasting sample. The median postprandial increase in particle concentration in the fresh samples was 61%.

Also, a significant increase in mean particle diameter was observed. The median of the mean particle diameters in all participants was 62 nm in the fasting samples, while in the postprandial samples it was 93 nm.

Tunable resistive pulse sensing

Also with TRPS we measured higher particle concentrations in the postprandial as compared to the fasting PFP in all study participants, and the changes were much more pronounced than those observed with NTA. Thus, the median postprandial change in particle concentration in the fresh samples was a 14-fold increase. No change in mean particle diameter was observed.

Lipid levels and their correlation with particle concentrations

The TG concentration displayed a statistically significant postprandial increase, with the median concentration increasing from 1.0 to 1.2 mmol/L after food ingestion. Cholesterol levels were not significantly changed.

In the postprandial samples TG levels significantly correlated with particle concentrations measured by NTA (r_s = 0.85) as well as TRPS (r_s = 0.80). In the fasting samples TG levels more moderately yet statistically significantly correlated with particle concentrations measured by NTA (r_s = 0.50), while there was no significant correlation between TG levels and particle levels measured by TRPS.

Cholesterol levels did not significantly correlate with particle concentrations measured by neither NTA nor TRPS.

FRESH VERSUS THAWN SAMPLES

Nanoparticle tracking analysis

Significantly higher particle concentrations were seen after one freeze-thaw cycle. In the fasting PFP samples, one freeze-thaw cycle resulted in a median increase in particle concentration by 29 %. In the postprandial samples the median increase was 38 %. No changes in mean particle size was observed.

Tunable resistive pulse sensing

With TRPS, we observed a median increase in particle concentration by 32 % in the fasting samples after one freeze-thaw cycle. No change in concentration was observed after freezing and thawing the postprandial samples. With regard to mean particle diameter, a median postprandial increase by 11 % in the fasting samples and 10 % in the postprandial samples, was observed.

PFP FRACTIONS OBTAINED BY SIZE EXCLUSION CHROMATOGRAPHY

The particle populations in the fractions obtained after SEC were analyzed by NTA. In SEC fraction 9 and 10 obtained from fasting PFP the particle concentration was 4.1 x 10^{10} particles/mL and 3.5 x 10^{10} particles/mL, respectively. In fraction 9 and 10 a mean postprandial increase in particle concentration of 113 % and 62 %, respectively, was observed. Applying a freeze-thaw cycle on fraction 9 and 10 of fasting PFP resulted in an increase in particle concentration by 43 % and 24 %, respectively.

The mean particle diameter in was 74 nm in fresh fasting PFP samples before SEC, while in fraction 9 and 10 it was 81 and 80 nm, respectively. In fresh postprandial samples the mean particle diameter was 91 nm in PFP, 104 nm in fraction 9, and 83 nm in fraction 10. We did not observe a systematic effect of a freeze-thaw cycle on mean particle diameter.

STUDY 2

EFFECT OF BEAD PROCEDURE ON PARTICLE CONCENTRATION

Lipoprotein isolates

The bead procedure was applied on the diluted VLDL isolate and on the diluted LDL isolate, resulting in a decrease in particle concentration of 93 % and 91 %, respectively. In the VLDL isolate, particles with diameters up to 450 nm were observed and in the LDL isolate, particles with diameters up to 250 nm were observed. Thus, considerable amounts of particles larger than expected for VLDL and LDL particles were detected in the respective isolates.

Platelet free plasma

Applying the bead procedure on PFP samples resulted in a significantly reduced particle concentration. The median reduction in particle concentration was 62 %. Particle reduction was typically accompanied by an increase in mean particle diameter in the remaining particles. Thus, the median of the mean particle diameters increased from 54.7 nm to 91.7 nm.

We explored the possibility of applying the bead procedure on less diluted PFP than the 1:1000 and 1:500 dilutions that were used in the main study. We found that it was possible to go down to a 1:400 dilution of PFP and obtain results on particle reductions that were comparable to the reductions observed with the standard bead procedure, while particle reductions became increasingly less effective at lower dilutions than 1:400.

As in study 1, particle concentrations correlated significantly with TG concentrations but not with cholesterol levels.

EFFECT OF BEAD PROCEDURE ON APOLIPOPROTEIN B CONCENTRATION

Lipoprotein isolates

Measuring the ApoB content in the diluted lipoprotein isolates before and after performing the bead procedure, we observed a reduction in ApoB content by 97 % in the diluted VLDL isolate and a reduction by 69 % in the LDL isolate.

Platelet free plasma

The ApoB ELISA was applied on four PFP samples before and after the bead procedure. Reductions in ApoB content after the bead procedure varied between 93 % and 97 %.

EFFECT OF BEAD PROCEDURE ON EXTRACELLULAR VESICLE CONTENT

Applying the bead procedure on four PFP samples resulted in reductions in EV content as measured with the EV Array in all four samples. The median reduction was 21 %.

STUDY 3

FLOW CYTOMETRY

Fasting versus postprandial samples

The measured concentration of total submicron particles in PFP was markedly increased after the breakfast meal in all study participants. The median increase was 1385 %. Total submicron particle concentrations significantly correlated with TG levels in the postprandial state (r_s = 0.79), while in the fasting state, they did not.

A statistically significant postprandial increase in the concentration of PS-positive particles was observed, the median increase being 63 %. Also, levels of TF-positive, CD41-positive, CD146-positive, and CD62E-positive subtypes of PS-positive particles were significantly increased postprandially, the median increases being between 49 and 307 %. Furthermore, levels of TF-positive PS-negative events increased significantly after food consumption.

In vitro addition of lipoproteins to fasting samples

Four fasting PFP samples were spiked with LDL and parallelly with VLDL isolate. The measured concentrations of total submicron particles and total PS-positive submicron particles increased three- to four-fold. Subgroups staining positive for TF, CD41, CD146, or CD62E, increased by between 8 and 172 %. We also analyzed control samples, which underwent an identical procedure except from PFP being replaced by additional DPBS. After addition of LDL and VLDL to these control samples, substantial amounts of unstained submicron particles were measured, while no antibody-labeled subtypes of PS-positive events appeared upon spiking with neither LDL nor VLDL.

EXTRACELLULAR VESICLE ARRAY

Measurable amounts of CD41-positive EVs were detected in fasting samples from all study participants. PS-positive, CD146-positive, and TF-positive EVs were detected in fasting PFP from the majority of the study subjects while CD62E-positive EVs were undetectable in most of the samples. Using anti-CD63 antibodies as capturing agents fluorescence intensities below the detection limits were observed in nearly half of the samples, while anti-CD81 captured detectable amounts of EVs in all samples and anti-CD9 did so in PFP from 19 of 20 study participants.
No systematic differences were observed between EV levels measured in the fasting compared to the postprandial samples.

TISSUE FACTOR ASSAYS

While TF antigen was detected in all PFP samples, none of the PFP samples displayed measurable levels of TF activity. A statistically significant positive correlation between the TF ELISA and the additional EV Array-based method for TF-antigen measurement was found both in the fasting and, more markedly, in the postprandial samples samples (Pearson's r = 0.73). There were no systematic differences between TF antigen concentrations in the fasting as compared to the postprandial samples. TF antigen concentrations neither correlated with levels of TF-positive EVs as measured by EV Array nor concentrations of TF-positive PS-positive particles measured by FC. However, TF antigen concentrations measured with the EV Array-based method for TF-antigen measurement moderately correlated with total TF-positive events measured by FC (r_s = 0.46).

PROCOAGULANT PHOSPHOLIPID DEPENDENT CLOTTING TIME

No systematic changes in PPL-CT were observed when fasting and postprandial samples were compared. PPL-CT did not correlate with concentrations of PS-positive submicron particles measured by FC.

STUDY 4

No significant differences were found between the paroxysmal and persistent/permanent AF group, and in the following these two groups are combined and referred to as the AF patient group.
Nor were any significant differences observed between PFP samples from the first and the third tube of blood drawn from the LAA.

PLASMA LEVELS OF TISSUE FACTOR ANTIGEN

Significantly higher concentrations of TF antigen were measured in pre- as well as intraoperatively collected venous samples from AF patients compared with controls. The same tendency was observed in the samples drawn from the LAA, although this was not statistically significant (p = 0.07).
In general, there was a trend towards lower TF concentrations in the intraoperative

venous and LAA samples than in the preoperative venous samples. This difference was statistically significant when comparing levels in the preoperative venous with levels in the intraoperative venous samples in the AF patient group.

PLASMA LEVELS OF VON WILLEBRAND FACTOR

Higher vWF concentrations were present in the AF patient group than in the control group. This difference was statistically significant in the LAA samples as well as in the pre- and intraoperative venous samples. When comparing the LAA and the pre- and intraoperative samples within each of the two groups, no significant differences were found.

PLASMA LEVELS OF CELL FREE DEOXYRIBONUCLEIC ACID

In both the AF patient group and the control group significantly higher PFP levels of cf-DNA were meausured in the LAA samples and the intraoperative venous samples than in the preoperative venous samples. When comparing the AF patient group with the control group, no significant differences were observed.

PLASMA LEVELS OF PROCOAGULANT PHOSPHOLIPIDS

Neither when comparing the AF patient group with the control group nor when comparing the preoperative venous samples, the intraoperative venous samples, and the LAA samples any significant differences in PPL-CT were observed.

PLASMA LEVELS OF SUBMICRON PARTICLES

No significant differences in concentrations or mean diameter of submicron particles measured by NTA were observed neither when comparing the two groups nor when comparing preoperative venous samples, intraoperative venous samples, and LAA samples within each group. In accordance with the findings in study 1 and 2, particle concentrations correlated significantly with TG levels ($r_s = 0.82$). Moreover, in this study, particle concentrations correlated with total cholesterol ($r_s = 0.55$) and LDL cholesterol ($r_s = 0.52$).

PLASMA LEVELS OF EXTRACELLULAR VESICLES

No significant differences between EV levels in LAA blood as compared to venous blood were found in patients with or without AF.

Levels of EVs captured and detected by anti-tetraspanin antibodies were significantly higher in AF patients than in control patients in the intraoperative venous samples. A similar, but statistically insignificant, trend was seen in the preoperative venous samples ($p = 0.12$) and in the LAA samples ($p = 0.10$).

We observed significantly higher levels of tetraspanin-exposing EVs that were also annexin V-positive in AF patients as compared to control patients in the pre- as well as the intraoperative venous samples with the same but not statistically significant tendency ($p = 0.05$) observed in the LAA samples.

Levels of EVs captured by anti-CD146 or anti-CD206 antibodies combined with

detection by anti-tetraspanin antibodies were significantly higher in AF patients than in control patients in pre-, and intraoperative venous samples as well as LAA samples. Likewise, significantly higher levels of EVs measured with anti-CD31 as capturing antibody combined with detection by anti-tetraspanin antibodies were seen in AF patients than in controls with regard to the preoperative venous samples and the LAA samples, and in the intraoperative venous samples the same tendency was observed, although this was not statistically significant ($p = 0.11$). When capturing with anti-CD163, anti-CD14- ($p = 0.06$ in the preoperational samples in both cases) and to a lesser extent anti-CD41, anti-CD42a, or anti-CD42b ($p > 0.17$ in all cases) antibodies combined with detection by anti-tetraspanin antibodies was performed, a statistically insignificant tendency towards higher levels in the AF patient group than in the control group was also observed.

PLASMA LEVELS OF TISSUE FACTOR-BEARING EXTRACELLULAR VESICLES

No significant differences in levels of TF-bearing EVs in LAA blood as compared to venous blood were found patients with or without AF.
When EVs were captured by anti-TF antibodies of the TF9-10H10 clone combined with detection by anti-tetraspanin antibodies, significantly higher levels were found in the AF patient group as compared to the control group in the preoperative and intraoperative venous samples as well as the LAA samples. When the HTF-1 clone was used as capturing antibody the same tendency was seen in all three sample types, although it was only statistically significant in the preoperative venous samples.

When anti-CD14 antibodies were used as capturing agents combined with anti-TF antibodies as detecting agents, significantly higher EV levels were found in the preoperative venous samples and the LAA samples from AF patients as compared to controls, and the same trend, but statistically insignificant ($p = 0.09$) was observed in the intraoperative venous samples. A statistically insignificant trend towards higher EV levels in the AF patients than in the controls was also seen when capture by anti-CD31, anti-CD146, or anti-CD206 antibodies were combined with detection by anti-TF antibodies. When anti-CD42a, anti-CD42b, anti-CD62E, or anti-CD163 antibodies were used as capturing agents in combination with anti-TF as detecting agent, most PFP samples did not display a measurable signal. Anti-CD41 as capturing agent combined with detection by anti-TF antibodies resulted in a detectable signal in most samples, while no trend towards differences between the groups was observed.

DISCUSSION

In this PhD study we aimed to investigate the potential role of EVs in AF-related thrombogenicity. We hypothesized that elevated blood plasma levels of procoagulant EVs exist in AF patients as compared to subjects without AF. We furthermore hypothesized that elevated blood plasma levels of procoagulant EVs exist in the LAA in patients with AF. The theoretical rationale behind these hypotheses has been covered in the background section and summarized in the aims and hypothesis section.

The decision to investigate levels of procoagulant EVs in an AF patient group and a control group gave rise to considerations on how to measure EVs. Each method of EV isolation and analysis holds limitations[171]. Evaluation of the potential of EVs as biomarkers for thrombogenicity entails examination of existing methods for EV detection, including investigation of the susceptibility of EV measurement to interference from biological variables such as lipoprotein levels. Taking a clinical biochemical approach, we therefore aimed to gain hands-on experience and address some of the challenges related to EV analysis through establishment and evaluation of methods for EV analysis. Operating in a field that is marked by inconsistent nomenclature[128] and lack of methodological standardization[123], the task of method establishment and evaluation can appear to be an abyss of uncertainty. We had to settle for elucidation of a small selection of issues related to EV analysis, and we chose to put a particular focus on interference from lipoproteins.

MEASUREMENT OF EXTRACELLULAR VESICLES

STUDY 1

Analytical issues

Analytical linearity

The data in study 1 indicated analytical linearity for NTA within a concentration range that allowed most PFP samples to be diluted into the linear range by a 1:1000 dilution and in all other cases to identify a suitable dilution factor by applying one extra dilution procedure. PFP did not need to be diluted to be within the linear range of TRPS, but dilution by a factor of 2.5 or 5.0 was preferable in order to avoid nanopore clogging. Further dilution of PFP is also a possibility with TRPS, based on the linearity study, and would further reduce the risk of nanopore clogging but would also extend analysis time and thus increase the possibility of conformational changes in the nanopore itself and a resulting change in baseline current during analysis. Our linearity data were obtained by measuring a dilution series of PFP and agreed well with findings by others on NTA and TRPS linearity in studies on polystyrene bead solutions[164,211].

Analytical variation

Taken together, the CVs in the analytical variation study indicated acceptable precision. While the between-day variation study on PFP displayed a rather high CV, this was not the case with the additional between-day variation study on polystyrene microspheres, which may indicate that the between-day data on PFP were influenced by preanalytical variation that may have to do with freezing or thawing the sample.

Technical considerations

Because of the lack of an ideal reference material for NTA of biological particles[196] it is not possible to apply definite accuracy testing of submicron particles in a PFP sample. Despite this inherent lack of opportunity to measure absolute concentrations, we consider it feasible to perform comparison studies in which particle levels in different types of samples such as fresh versus thawed, or fasting versus postprandial samples are compared. This viewpoint has been supported in a recent publication on NTA[212]. Throughout study 1, NTA displayed robustness. TRPS, on the other hand, is complicated by the tendency of the nanopore to undergo changes, possibly due to repeated stretch of the thermoplastic polyurethane material, and by a tendency of nanopore clogging especially when analyzing modestly diluted PFP. This problem, which has also been noted by other investigators[179], entails time-consuming troubleshooting procedures and hampers the practical applicability of the method in the laboratory. In particular, we experienced instability of the nanopores during the start-up of the additional study on EV-enriched SEC fractions, which eventually resulted in the decision not to include TRPS in that substudy.

Biological variation of submicron particle levels

In the light of the limitations regarding NTA's ability to measure exact concentrations of biological submicron particles, determination of the absolute biological variation is not possible. Since concentration measurements with NTA depend considerably on camera and detection threshold settings[213], the estimated reference ranges only provide an indication of the submicron particle levels that can be expected when analyzing PFP with the chosen settings specified in the materials and methods section, using the LM10-HS and NanoSight NTA 2.3.

TRPS also holds limitations with regard to particle concentration determination. The size range of particles that can be measured depends on the given nanopore and since the smallest particles present in PFP cannot be measured with any of the nanopores available, the biological variation in particle concentration that we measured by TRPS applies to the exact size range measured with the chosen nanopore. Given that a considerable amount of particles in PFP have diameters below the minimum detection limit of the qNano system[164,178], the method cannot provide an exhaustive overview of the particles in a PFP sample but nevertheless, like NTA, it may be used for comparison of the relative particle content within a given size range in different sample types.

The presented reference intervals for submicron particle concentrations in PFP

measured by NTA and TRPS with the given equipment and settings are to some extent in line with findings by other investigators reporting particle concentrations in PFP of 1 - 5 x 10^{12} particles/mL measured with NTA[195] and particle concentrations in non-purified plasma of approximately 2 x 10^{8} particles/mL measured with TRPS, applying an NP200 nanopore[179].
The estimated intra-individual variation measured with TRPS and especially NTA was considerable, displaying within-subject CVs similar to e.g. those of total bilirubin and conjugated bilirubin, respectively[214].
Regarding the biological variation related to prandial state, our data supported the hypothesis that TG-rich lipoproteins appearing in the postprandial state increase particle numbers measured by NTA and TRPS. The fact that NTA has a substantially lower detection limit with regard to particle diameter plausibly explains why the relative postprandial increase in measured particle concentration is much more extensive in TRPS than in NTA, as the particles appearing upon food intake obviously contribute relatively more to the population of larger particles than smaller particles. The differences in lower detection limit between the two methods also explains why NTA shows an increase in mean particle diameter in the postprandial samples when NTA is applied, while TRPS does not despite the marked increase in particle concentration demonstrated by TRPS. The diameters of many chylomicrons[202,204] and a smaller portion of the postprandially appearing VLDLs[205,206] correspond to the size range of TRPS with an NP200 nanopore. The finding of stronger correlation between particle concentration and TG concentration in the postprandial as compared to the fasting state also suggests that postprandially appearing lipoproteins represent a considerable part of the higher particle numbers measured after food intake. However, since indications exist that EV concentrations in plasma may also increase in the postprandial state[110,134,135,215], part of the measured postprandial elevation of particle concentrations may be accounted for by EVs. Applying NTA on specific SEC fractions which should be relatively enriched in EVs as compared to HDLs and proteins[192] showed postprandial changes in particle concentration and size levels that were similar to the changes observed in the original PFP samples.

Effect of a freeze-thaw cycle on submicron particle levels

While mean particle diameter measured by NTA was not significantly affected by freezing or thawing the samples, the median increase in particle concentration by about 30 % that was observed in both NTA and TRPS measurements, indicates that it may be preferable to analyze fresh samples. Therefore, the NTA measurements in study 4 were performed on fresh samples.

Link to study 2

While NTA and TRPS enable detection of *particles* smaller than the ones that can be measured by conventional FC, the indication of a considerable contribution of lipoproteins to the measured particle numbers when applying the methods directly on PFP, questions the value of using NTA and TRPS for measurement of *EVs* in PFP. Apart from allowing for a wider size range of particles than TRPS, NTA was

considerably more robust than TRPS in daily use in the laboratory.
To further explore the interference of lipoproteins on NTA-based measurement of EV levels in PFP, we performed study 2, in which we tested the potential of lipoprotein removal by the bead procedure.

STUDY 2

Effect of the bead procedure on lipoprotein solutions

The bead procedure reduced particle concentrations in VLDL and LDL solutions to a level, which was lower than the linear range of NTA measurements and also reduced the ApoB concentration in the VLDL solution by 97 %. However, it did not reduce the ApoB concentration in the LDL solution quite as effectively as 31 % remained after the bead procedure. The explanation to this discrepancy between the extent of measured particle removal and ApoB removal from the LDL solution may be the overlap between the size range of LDL particles[206] and the lower size detection limit in NTA[173-175]. Thus a fraction of the LDL particles may not be detectable by NTA and the amount of anti-ApoB antibodies used in the bead procedure may not have been sufficient to capture all LDL particles. If this is the explanation, it appears that especially the smaller LDL particles have a tendency to dodge the antibody-coated beads.

Effect of the bead procedure on platelet free plasma

When it came to the PFP samples, the bead procedure prompted effective ApoB removal, which was accompanied by a median reduction in particle concentration by 62 %. Also, we observed an unwanted side effect in the form of a variable reduction of EV content. This may result from LDL particles binding to EVs, a phenomenon that has been demonstrated in vitro[191]. Furthermore, we observed a general increase in mean particle diameter after the bead procedure. It could be speculated that this results from crosslinking of potentially unbound anti-ApoB antibodies to ApoB and formation of aggregates between lipoproteins and anti-ApoB antibodies. Alternatively, it may have to do with challenges of precise NTA on polydisperse samples[195] as lipoprotein depletion by the bead procedure may induce a change in the relative number of larger particles in the sample.
Taken together, our results indicate that removal of ApoB-bearing lipoproteins from PFP can be achieved by use of anti-ApoB antibody-coated magnetic beads, while other effects on the samples, including variable EV-removal and an increase in the measured amount of larger particles are also observed. It should also be mentioned that even after removal of ApoB-exposing lipoproteins, HDL particles in PFP may interfere with NTA measurements of EVs. It may be possible to further develop and optimize the bead procedure, e.g. via testing other antibody clones. While HDL particles are expectedly too small to be measured by NTA, it is conceivable that HDL particles can form aggregates with each other or with EVs, thus affecting concentration and size distribution results. It may be possible to remove HDL particles from PFP by a similar procedure, by substituting or supplementing anti-ApoB antibodies with anti-ApoA-I antibodies.

Link to study 3

While NTA provides robust measurement of submicron particles in PFP, it does not enable detailed characterization of the detected particles, such as identification of EV markers and thus distinction of EVs from lipoproteins. The interference of lipoproteins may be reduced by the bead procedure presented in study 2, but the EV population is also affected, and moreover protein aggregates[123] in the EV size range may represent a non-negligable source of interference. In this PhD project, we had a particular interest in the possibility of measurement of TF-bearing EVs, which we wanted to apply in study 4. Therefore, we conducted study 3, in which we tested FC- and EV Array-based measurement of EVs exposing TF and markers of cellular origin and the influence of prandial state on the results.

STUDY 3

Flow cytometry

The 1385 % increase in total particle concentration measured by FC in the postprandial phase agreed well with the 14-fold increase in particle concentration measured with TRPS in study 1, conceivably because FC and TRPS detect particles in roughly similar size ranges. While the postprandial increase in total particles can be explained by the appearance of chylomicrons in the postprandial phase, the significant increase in PS-positive particles and all measured subtypes as well as PS-negative TF-positive particles prompts further consideration. Although elevated postprandial levels of EVs derived from endothelial cells[134] and platelets[135] have previously been reported, we speculated that an artefactual effect of postprandially appearing lipoproteins may account for at least some of the increase in measured levels of antigen-exposing PS-positive particles. The finding that in vitro addition of commercially acquired LDL particles or VLDL particles to fasting PFP also resulted in increased levels of subtypes of PS-positive particles and subtypes staining positive for TF, CD41, CD146, or CD62E supported that theory. As many EVs below the lower size limit of detection by FC exist, a possible mechanism of lipoprotein interference could be simultaneous passing of one or more EVs too small to be detected by themselves and one or more lipoproteins through the detecting system of the flow cytometer. This could imaginably result in a variant of swarm detection in which lipoproteins act as vehicles for vesicles to become 'visible' by FC. The tendency of lipoproteins and EVs to pass the laser beam in the flow cytometer simultaneously may be enhanced by the tendency of LDLs to adhere to EVs in vitro[191].

Extracellular vesicle Array

While it has been stated that cells do not release TF-bearing EVs under non-pathological conditions[216], the existence of TF-bearing EVs in the circulation in healthy persons has been described[150,217]. Our EV Array results also indicated the presence of TF-bearing EVs in PFP from healthy individuals. Neither the levels of TF-bearing EVs nor any other measured subtype of EVs measured by EV Array were significantly changed by food intake and thus seemingly not considerable affected by lipoprotein content in the samples. The observation that in many samples

capturing with anti-CD63 antibodies did not result in a detectable fluorescence intensity are in line with findings by Pugholm et al.[218] who suggested that CD63 may be a poor marker for small EVs in general.

Tissue factor assays

The absence of TF activity levels above the limit of detection in any PFP samples is in line with the findings by others on a healthy population[111,219]. While measurement of TF antigen may not exclusively measure active TF, our results did not indicate systematic changes in TF antigen levels in the postprandial as compared to the fasting state. Neither did levels of TF-positive EVs measured by EV Array change postprandially. Taken together, these findings and the data on PFP samples spiked with lipoproteins, are in accordance with the theory of a lipoprotein-induced swarm detection-like interference on the FC results on TF-positive events.

Procoagulant phospholipid dependent clotting time

While the number of PS-positive events measured by FC increased postprandially this was not reflected in shortened PPL-CT, which is in accordance with findings by Tushuizen et al.[110] and furthermore with the suggestion that the increased concentrations of PS-positive particles measured by FC may reflect increased interference from lipoproteins rather than increased EV levels in the postprandial state.

Link to study 4

In study 4, we applied the EV Array and the TF ELISA that we gained experience with in study 3. Aware of the fact that different clones of TF antibody have different TF binding characteristics[220], we applied two different clones of TF as capturing agents in the EV Array in study 4. We also intended to apply FC on the PFP samples in study 4, and the Department of Clinical Biochemistry, Aalborg University Hospital, recently established small particle FC, enabling measurement of particles with a diameter down to 100 nm in PFP[221]. However, due to recurring technical problems, it has not yet been possible to apply that method on the AF patient and control samples.

ATRIAL FIBRILLATION AND PROCOAGULANT AGENTS

STUDY 4

Venous versus left atrial appendage blood samples

Our data did not support the hypothesis on elevated levels of procoagulant EVs in the LAA blood as compared to venous blood in patients with AF. However, a mean luminal LAA volume of 5.4 mL with a standard deviation of 3.7 mL in patients with nonvalvular AF has been reported[103]. Thus, as a blood volume of 3.5 mL was drawn into the first tube of blood collected from the LAAs in our study, some admixture of blood from the rest of the circulation has in all probability occurred in at least some

of the LAA blood samplings from the AF patients, and we cannot exclude the possibility that this has camouflaged differences between the sample types.

Pre- versus intraoperative blood samples

In a recent review it was noted that circulating cf-DNA can origin from varied sources, including cell death(46), and in our study the higher intra- than preoperative levels plausibly result from sternotomy-induced cell damage and possibly also sterile inflammation.

The lower TF antigen levels in the intra- as compared to the preoperative PFP samples may conceivably result from TF consumption or a configurational change in the blood-borne TF upon activation of the coagulation system following sternotomy.

Atrial fibrillation patients versus controls

Extracellular vesicle levels

The EV Array results on tetraspanin-positive, annexin V-positive, CD31-positive, CD146-positive, and CD206-positive EVs, indicate that AF is associated with increased levels of EVs, including subtypes derived from endothelial cells, monocytes, and macrophages. The similar, although statistically insignificant trend observed for CD14-exposing EVs and CD163-exposing EVs underpin the suggestion that EVs of the monocyte-macrophage lineage are more abundant in AF patients, while levels of EVs positive for the additional endothelial cell-marker, CD62E did not differ between AF patients and controls. To our knowledge, levels of EVs derived from leukocytes in AF patients as compared to patients wihout AF have not previously been investigated. Our findings on annexin V-positive EVs are in line with the findings by Ederhy et al.[161] on higher plasma levels of PS-positive EVs in AF patients as compared to a control group *without* and a control group *with* cardiovascular risk factors. Ederhy et al. also demonstrated higher levels of CD31-positive EVs in nonvalvular AF patients as compared to a control without cardiovascular risk factors while the levels in the nonvalvular AF patients were similar to a control group with cardiovascular risk factors. With regard to platelet-derived EVs, higher levels have been reported in blood from AF patients than controls without cardiovascular risk factors[160-162], while levels were similar when comparing AF patients to a control group with cardiovascular risk factors[160,161].

Our data on TF-positive EVs, both when applying the TF9-10H10 clone and the HTF-1 clone as capturing agents, clearly indicate that AF is associated with higher concentrations of TF-bearing EVs, which probably at least partly explains the elevated levels of blood-borne TF antigen in AF patients[102]. Furthermore, our data on EVs positive for both CD14 and TF suggest that the increased levels of TF-bearing EVs in AF patients to some extent depends on release of TF-bearing EVs from cells of the monocyte-macrophage lineage.

Additional analyses

Our demonstration of significantly higher concentrations of vWF in AF patients than controls are in line with previous reported data by other investigators[87].

Our data on blood-borne TF levels as measured by ELISA in AF patients complement previous findings by Chung et al., who demonstrated higher levels of TF in AF patients than in a healthy control group but similar levels of TF in AF patients and a control group with coronary artery disease[102], not specifying if the AF group included both valvular and nonvalvular AF patients.

The absence of differences between cf-DNA levels in AF patients as compared to controls suggest that NETs are not an important factor in AF-related thrombogenicity. Nor did we find differences in PPL-CT levels despite the observed differences in EV levels. Thus, it is a recurring observation that no plain relationship between EV levels and amounts of measurable PPLs, such as PS, exists[110].

The correlation between the NTA-based particle counts and the lipid levels substantiate the findings in study 1 and study 2 regarding considerable influence of lipoprotein content on submicron particle measurement.

CONCLUSIONS

In study 1, we found that the performance of NTA as well as TRPS was acceptable regarding analytical linearity and variation, while daily use of TRPS was cumbersome, primarily due to nanopore instability. Applying NTA and TRPS on PFP, an appreciable inter- and intra-individual variation in particle concentration and mean particle size was observed. Particle concentration and size distribution were affected by a freeze-thaw cycle and substantially affected by food intake, the latter probably due to appearance of TG-rich lipoproteins in the postprandial state. Correlation analysis in study 1, as well as study 2 and 4, indicated that particle concentrations in fasting PFP measured by NTA were influenced by lipoprotein content.

In study 2, we demonstrated that clearing of lipoproteins from diluted PFP by application of anti-ApoB antibody-coated magnetic beads prior to NTA appears to be achievable, while decreased EV content and increased levels of relatively large particles measured by NTA were also observed after application of the bead procedure.

In study 3, we demonstrated that food intake prompted elevated levels of PS-positive particles, including TF-, CD41- , CD146-, and CD62E-positive subtypes, in PFP measured by FC. A similar increase in PS- and antigen marker-positive particles was observed upon in vitro addition of LDL or VLDL to fasting PFP, indicating that plasma lipoproteins affect FC measurements of these analytes. Thus, the interplay between EVs and lipoproteins should be considered when applying FC-based measurement of PS-positive particles, and subgroups exposing specific antigens, as an approach to EV measurement. EV levels as measured by EV Array were not changed by food consumption.

In study 4, we demonstrated elevated levels of vWF, TF, and EVs positive for markers of leukocyte and endothelial cell origin in AF patients as compared to controls. Furthermore, we observed higher levels of TF-bearing EVs, including TF-bearing EVs

of the monocyte-macrophage lineage, in AF patients than in controls, suggesting that AF-related release of TF-bearing EVs at least partially explains the increased levels of circulating TF antigen in AF patients. We did not find different levels of EVs, including TF-bearing EVs, in LAA blood as compared to venous blood in patients with AF. EVs are considered to possess procoagulant capacity and may be mechanistically involved in increased thrombogenicity in AF patients.

PERSPECTIVES

Science never solves a problem without creating ten more.

George Bernard Shaw (1856-1950)[222].

The provided data on method performance, possible sources of error in EV analysis, proof of concept of antibody-mediated lipoprotein removal, and elevated levels of TF-bearing EVs in AF patients may give rise to more questions than answers, but hopefully in that way, they represent useful stepping stones to scientific progress that can eventually be of benefit to patients.
EV research is still regarded as a young field[122,171], and the need for standardization of methodology and technology is increasingly recognized. Development of improved methods for EV purification and analysis are expected to help move the field forward[122], and ISEV and the International Society on Thrombosis and Haemostasis recently joined forces to standardize FC-based EV detection[171]. Our results in Study 3 supplemented the findings by Sódar et al.[191], indicating that previously unacknowledged challenges of lipoprotein interference on FC-based EV analysis on blood plasma may with advantage be taken into consideration in that connection.
It may be possible to refine our described bead procedure with regard to choice of antibody clones, incubation periods, and washing steps in order to remove lipoproteins and protein aggregrates without introducing a considerable EV loss. To broaden the range of methods that could be applied for EV counting upon lipoprotein removal to include e.g. FC and TRPS, it would be necessary to design a procedure enabling effective lipoprotein removal from plasma diluted less than 1:400. Regarding TRPS, however, in our experience it would also be necessary to improve the robustness of the method in order to apply it for routine use on plasma samples.

Study 4 of this PhD project is the first study to investigate levels of TF-bearing EVs in AF patients. There is an increasing focus on distinction between patients who do and do not require oral anticoagulation[109] and on elucidation of which anticoagulation strategies suit different subgroups of AF patients best[223]. Blood plasma biomarkers may hold potential as a supplement to the CHA_2DS_2-VASc score. While vWF levels have been demonstrated to provide some additional prognostic information with regard to cardiovascular events and mortality, the absolute impact on decision-making was considered too small to prioritize inclusion of vWF measurements in the

strategy for risk prediction[224]. Study 4 substantiated the finding by Chung et al.[102] on elevated levels of blood-borne TF in AF patients and, for the first time, indicated elevated levels of TF-bearing EVs, including CD14-positive TF-bearing EVs in AF patients. As procoagulant capacity of these EVs may play a mechanistic role in thrombogenicity in AF patients, TF-bearing EVs may represent relevant candidates for exploration in prospective studies evaluating their potential as predictors of thromboembolic events in AF patients. Moreover, TF-positive EVs have been suggested as potential targets for future anticoagulants[48]. Our data suggest that AF patients may represent a group, which could benefit from such therapeutic advancement.

ENGLISH SUMMARY

Atrial fibrillation (AF) is a prevalent cardiac arrhythmia that significantly increases the risk of thrombus formation in the left atrial appendage (LAA) of the heart and subsequent thromboembolism. The mechanisms by which AF promotes thrombus formation include local blood stasis in the LAA, while inflammatory processes and elevated blood levels of procoagulant molecules may also be involved. In recent years, extracellular vesicles (EVs) have been increasingly considered as potential prothrombotic agents. EVs are lipid bilayer membrane-bounded particles released from various cell types as a response to physiological or pathological stimuli. The external membrane of an EV can facilitate accumulation of blood coagulation factors and, furthermore, it can contain tissue factor (TF), the principal initiator of blood clotting. Measurement and characterization of EVs in the blood is challenging as they are delicate and heterogenous analytes, coexisting with other blood components, such as lipoproteins, that may interfere with the analysis. In the four studies included in this PhD thesis, methods for EV detection and characterization were tested, addressing preanalytical and analytical challenges with a particular focus on interference from lipoproteins. Furthermore, venous and LAA blood levels of components with a thrombogenic potential, including TF-bearing extracellular vesicles, were evaluated in a group of AF patients and a control group.

In study 1, we found acceptable analytical performance of two methods for particle counting, namely nanoparticle tracking analysis and tunable resistive pulse sensing. Appreciable inter- and intra-individual variation in particle concentration and mean particle size in platelet free plasma (PFP) from healthy individuals was observed. Particle concentration and size distribution were affected by a freeze-thaw cycle and, more markedly, by food intake, probably due to appearance of lipoproteins in the postprandial state. Study 2 showed that it was possible to remove lipoproteins from diluted PFP by application of anti-apolipoprotein B antibody-coated magnetic beads. However, this 'bead procedure' also reduced the EV content as semiquantified by EV Array, which is a protein microarray-based method for EV analysis. In study 3, we demonstrated that plasma content of lipoproteins and, accordingly, prandial state affected flow cytometry measurements of particles staining positive for TF as well as for general and cell type specific EV markers, which indicates problematics of equating these particles with EVs. On the other hand, EV content as measured by EV Array was not affected by food intake. In study 4, we found elevated blood levels of TF antigen in AF patients compared to controls, which was in line with a previous study. A novel finding in our study was higher levels of TF-bearing EVs, including TF-bearing EVs of the monocyte-macrophage lineage, in AF patients than in controls as measured by EV Array. No differences between venous and LAA blood composition were detected. The results indicate that AF-related release of TF-bearing EVs at least partially explains the increased levels of circulating TF in AF patients. TF-bearing EVs may play a role in the increased thrombogenicity in AF patients and may hold potential as predictive markers of AF-related thromboembolism and as targets of future anticoagulants for the benefit of AF patients.

DANSK RESUMÉ

Atrieflimren (AF) er en hyppigt forekommende hjerterytmeforstyrrelse, som øger risikoen for trombose i hjertets venstre aurikel og tromboembolisme markant. En kendt mekanisme bag trombedannelsen er stase af blodet i venstre aurikel, mens inflammatoriske processer og øget koncentration af prokoagulante komponenter i blodet er mulige medvirkende årsager. I disse år er der stigende opmærksomhed på ekstracellulære vesikler (EV) som potentielle trombogene faktorer. EV er partikler med en dobbeltlaget lipidmembran, der udskilles af forskellige celletyper som respons på fysiologiske eller patologiske stimuli. Blodets koagulationsfaktorer kan komme i indbyrdes kontakt på overfladen af vesikelmembranen, som desuden kan indeholde proteinet tissue factor (TF), som er den primære initiator af koagulationskaskaden. Måling og karakteristik af EV i blodet er kompliceret pga. vesiklernes skrøbelighed og heterogenitet samt tilstedeværelsen af interfererende blodkomponenter som for eksempel lipoproteiner. I dette ph.d.-projekt, som omfatter fire studier, evaluerede vi metoder til måling og karakteristik af EV med vægt på problematikken vedrørende interferens fra lipoproteiner. Desuden sammenlignede vi niveauerne af en række potentielt trombogene komponenter, herunder TF-bærende EV, i veneblod samt i blod udtrukket fra venstre aurikel i en gruppe AF-patienter og en kontrolgruppe.

I studie 1 fandt vi en acceptabel analytisk præcision for to forskellige metoder til partikeltælling betegnet henholdsvis nanoparticle tracking analysis og tunable resistive pulse sensing. Desuden fandt vi en betydelig inter- og intraindividuel variation i partikelkoncentration og middelpartikelstørrelse i blodplasma hos raske personer. Partikelkoncentrationen og -størrelsesfordelingen var påvirkelig af frysning af plasma før analyse og mere markant påvirkelig af fødeindtag, sandsynligvis pga. en øget postprandial koncentration af lipoproteiner. I studie 2 viste vi, at lipoproteiner kan fjernes fra fortyndet plasma vha. magnetiske partikler med antistoffer mod apolipoprotein B, men også at denne procedure reducerer EV-indholdet i plasma målt med EV Array, som er en protein microarray-baseret metode til EV-semikvantificering. I studie 3 demonstrerede vi, at plasmas indhold af lipoproteiner påvirker flowcytometriske målinger af partikler med positivt signal for TF og for generelle og cellespecifikke EV-markører, hvilket gør det problematisk at anvende disse målinger til kvantificering af EV. EV-semikvantificering vha. EV Array blev derimod ikke påvirket af fødeindtag. I studie 4 fandt vi, i lighed med et tidligere studie, at AF-patienter havde højere plasmakoncentration af TF end kontrolpatienter. Desuden fandt vi, at AF-patienter havde et større indhold af TF-bærende EV i blodet, herunder TF-bærende EV deriveret fra monocyt-makrofag-cellelinjen, hvilket ikke tidligere er beskrevet. Vi fandt ikke forskelle mellem vene- og aurikelblod. Resultaterne indikerer, at AF-relateret frigivelse af TF-bærende EV er medvirkende årsag til en øget koncentration af cirkulerende TF hos AF-patienter. TF-bærende EV er en mulig medvirkende faktor i AF-relateret trombogenicitet og kan have potentiale som prædiktiv markør for tromboembolisme og som angrebsmål for fremtidige antikoagulantia til AF-patienter.

ABBREVIATIONS

ADP	adenosine diphosphate
AF	atrial fibrillation
ApoA-I	apolipoprotein A-I
ApoB	apolipoprotein B
asTF	alternatively spliced tissue factor
ATP	adenosine triphosphate
CD	cluster of differentiation
CV	coefficient of variation
DNA	deoxyribonucleic acid
DOAC	direct oral anticoagulant
DPBS	Dulbecco's Phosphate Buffered Saline
ECG	electrocardiography
EPCR	endothelial cell protein C receptor
ESC	European Society of Cardiology
ESCRT	endosomal sorting complex required for transport
EV	extracellular vesicle
FC	flow cytometry
FC region	fragment crystallizable region
FII	coagulation factor II
FIII	coagulation factor III
FIX	coagulation factor IX
flTF	full-length tissue factor
FSC	forward scatter
FV	coagulation factor V
FVII	coagulation factor VII
FVIII	coagulation factor VIII
FX	coagulation factor X
FXI	coagulation factor XI
FXII	coagulation factor XII
FXIII	coagulation factor XIII
GP	glycoprotein
HDL	high density lipoprotein
IDL	intermediate density lipoprotein
ILV	intraluminal vesicle
ISAC	International Society on Advancement of Cytometry
ISEV	International Society of Extracellular Vesicles
LA	left atrium
LDL	low density lipoprotein
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LAA	left atrial appendage
MV	microvesicle

MVB	multivesiclar body
NET	neutrophil extracellular trap
NO	nitric oxide
NTA	nanoparticle tracking analysis
OAC	oral anticoagulant therapy
PAI-1	plasminogen activator inhibitor-1
PAI-2	plasminogen activator inhibitor-2
PE	phycoerythrin
PFP	platelet free plasma
PPL	procoagulant phospholipid
PPL-CT	procoagulant phospholipid dependent clotting time
PS	phosphatidylserine
PSGL-1	P-selectin glycoprotein-1
RNA	ribonucleic acid
RAA	right atrial appendage
SEC	size exclusion chromatography
SSC	side scatter
TAFI	thrombin activated fibrinolysis inhibitor
TEM	tetraspanin-enriched microdomain
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TG	triglyceride
TM	thrombomodulin
TNF-α	tumor necrosis factor alpha
tPA	tissue plasminogen activator
TRL	Triglyceride-rich lipoprotein
TRPS	tunable resistive pulse sensing
TXA2	thromboxane A2
uPA	urokinase type plasminogen activator
VEGF	vascular endothelial growth factor
VKA	vitamin K antagonist
VLDL	very low density lipoprotein
vWF	von Willebrand factor

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SUMMARY

Atrial fibrillation is a prevalent cardiac arrhythmia that significantly increases the risk of thrombus formation in the left atrial appendage of the heart and subsequent thromboembolism. The mechanisms by which atrial fibrillation promotes thrombus formation may involve elevated blood levels of procoagulant molecules. In recent years, extracellular vesicles have attracted growing attention as potential prothrombotic agents. Extracellular vesicles are lipid bilayer membrane-bounded particles released from cells as a response to physiological or pathological stimuli. Extracellular vesicles can facilitate accumulation of blood coagulation factors and they can contain tissue factor, the principal initiator of blood clotting. Measurement of extracellular vesicles in blood is challenging as they are delicate and heterogenous analytes, coexisting with other blood components that may interfere with the analysis. In this PhD project, methods for extracellular vesicle detection and characterization were tested, addressing preanalytical and analytical challenges with a particular focus on interference from lipoproteins. Furthermore, venous and left atrial appendage blood levels of components with a thrombogenic potential, including tissue factor-bearing extracellular vesicles, were evaluated in a group of atrial fibrillation patients and a control group.

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